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13. ABSTRACT (Maximum 200 Words) In glandular organs, a precancerous state precedes invasive carcinoma. In the breast this state is recognized as DCIS and consists of an epithelial cell proliferation confined by myoepithelial cells. Our laboratory has established cell lines/xenografts of myoepithelial cells from benign breast and salivary myoepithelial tumors. These lines/xenografts express a suppressive phenotype. Our myoepithelial cell lines inhibit invasion and motility of breast carcinoma lines <i>in vitro</i> largely through maspin. The overall hypothesis of this proposal was how does myoepithelial maspin regulate breast (DCIS) carcinoma progression and can its detection in fine needle aspirates (FNA) and in ductal fluid aid diagnosis and screening? The first aim addressed the mechanism of maspin's inhibition of breast carcinoma invasion: We have shown that myoepithelial maspin does bind to plasma membranes of carcinoma cells and inhibit a pathway involved in cellular locomotion. The second aim utilized maspin antibodies on FNA to identify the myoepithelial component and we have demonstrated that this approach is successful. The third aim investigated the levels of maspin in nipple aspirates, in ductal lavage fluid and saliva. We have shown that maspin is both a tumor marker as well as a surrogate intermediate end point marker. Studies are planned in year 02 to extend our findings in each of these aims.				
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INTRODUCTION

In many glandular organs, a precancerous state is thought to exist which precedes the development of frankly invasive carcinoma. In the breast this state is recognized as DCIS and consists of a proliferation of epithelial luminal cells confined by myoepithelial cells within the ductal system (1-7). CGH and LOH studies have failed to demonstrate a difference between DCIS and invasive breast carcinoma. Our laboratory has recently demonstrated that paracrine regulation of this transition by myoepithelial cells may be the main determinant of this important step in human breast carcinoma progression. Because of their close proximity to precancerous lesions, myoepithelial cells would be expected to exert important paracrine influences on these processes. Myoepithelial cells of the breast differ from ductal cells in many ways: they lack ER- α , and its downstream genes; they synthesize the adjacent basement membrane; they rarely proliferate or fully transform and give rise rarely only to low grade benign neoplasms. Myoepithelial cells are present around normal ducts and precancerous proliferations but are absent in invasive carcinoma. Our laboratory has established immortalized myoepithelial cell lines and xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3) and breast (HMS-4,5) (8-23). These cell lines and xenografts express identical myoepithelial markers as their *in situ* counterparts. Our myoepithelial cell lines and xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α -1 antitrypsin, an unidentified perhaps novel 31-33 kDa trypsin inhibitor (24), thrombospondin-1, soluble bFGF receptors, and maspin (25-34). The human myoepithelial cell lines, HMS-1, HMS-3, HMS-4, HMS-5 inhibit both ER-positive and ER-negative breast carcinoma invasion (down to 42% \pm 7% of control) ($p < .05$) and in CM assays (down to 30% \pm 8% of control) ($p < .01$).

The anti-invasive effects of HMS-1, HMS-3, HMS-4, HMS-5 and HMS-6 can be enhanced by phorbol 12-myristate 13-acetate (PMA) (down to 2% \pm 1% of control) and abolished by dexamethasone (up to 95% \pm 5% of control) ($p < .01$). Therefore with the appropriate pharmacological treatment, the myoepithelial cells do not only partially inhibit invasion --- they in fact near-totally eliminate it. PMA treatment causes an immediate and sustained release of maspin, a recently identified serpin. Immunoprecipitation of maspin from this CM nearly abolishes this anti-invasive effect. Adding purified native myoepithelial maspin to unconditioned media also results in a dramatic anti-invasive effect. Maspin exerts a similar inhibitory effect on breast carcinoma cell motility as noted by us using native maspin and others using both recombinant bacterial maspin and recombinant insect maspin(i) produced in Baculovirus-infected insect cells (35). Our findings suggest that myoepithelial-secreted maspin functions as a paracrine tumor suppressor, which may inhibit *in vivo* the progression of DCIS to invasive breast carcinoma.

The observation that myoepithelial cells express and secrete maspin has, in addition to these biological implications, important potential practical applications. Fine needle aspiration cytology of the breast is a safe noninvasive technique for diagnosing breast cancer that is being used with increasing frequency in older women and women with comorbid disease who would benefit from being spared a more invasive and anesthesia-requiring procedure such as lumpectomy. Presently however there is no way on FNA of distinguishing invasive breast cancer cells from DCIS cells. This is because both types of cells appear cytologically identical and on

routine FNA there is no way to evaluate the cells in the anatomical context of the tissues. We feel however that if myoepithelial cells could be selectively identified on FNA then their presence would suggest the DCIS state since they would be expected to be aspirated along with the DCIS epithelial cells. Their absence on the smear would suggest that the malignant cells which were present were derived from invasive carcinoma cells which are devoid of surrounding myoepithelial cells *in vivo*. Perhaps the absolute number or density of myoepithelial cells would also be discriminating factors. Our laboratory has shown that the immunocytochemical demonstration of maspin reliably distinguishes myoepithelial cells from all epithelial cells (normal, DCIS, and invasive carcinoma). Furthermore our laboratory has detected maspin in ductal fluid of the breast obtained by both nipple suction and selective breast ductal cannulation (36). This maspin in ductal fluid is produced by myoepithelial cells *in vivo* and reflects the integrity of the normal ductal lobular unit. Conceivably reduced levels of maspin in ductal fluid may reflect either structural or functional compromise of the myoepithelial layer and may be seen in high risk v normal women and/or ducts with abnormal microcalcifications, precancerous or invasive histopathology.

BODY (STATEMENT OF WORK)

1. To investigate the mechanism of maspin's inhibition of breast carcinoma cell invasion and motility. - Months 1-36

A. Maspin binding to breast carcinoma cells/plasma membranes. - Months 1-6

B. Identification and characterization of a maspin binding protein.- Months 6-18

C. Maspin activation of a breast carcinoma cellular pathway.- Months 18-36

We have demonstrated during the first year of funding that purified myoepithelial maspin does indeed bind to plasma membranes of carcinoma cells via a membrane receptor which in turns inactivates a cellular pathway involved in cellular locomotion. We have also found that a number of different pharmacological agents can influence the secretion of maspin from myoepithelial cells and the myoepithelial cells' suppressive phenotype. These are contained in our publications (37,38,39,40).

2. To utilize antibodies to maspin on fine needle aspirate (FNA) specimens to quantitate the myoepithelial component and determine if this determination discriminates between DCIS and invasive breast carcinoma. - Months 12-24

We have demonstrated that antibodies to maspin (polyclonal and monoclonal) are the best discriminator between myoepithelial cells and epithelial cells and they can be used to quantitate the myoepithelial component.

3. To investigate the levels of maspin in ductal fluid obtained by the nipple suction approach (in high risk v normal women) and in ductal fluid obtained by selective ductal

cannulation and washings (ducts with and without microcalcifications from the same patient) and determine whether maspin levels abet screening and correlate with histological findings/ - Months 24-36

We have demonstrated that nipple aspirate fluids and ductal lavage fluids obtained by ductoscopy is rich in maspin as a surrogate intermediate end point marker that reflects the integrity of the ductal lobular unit. The methods used in these studies are contained in our publications (41,42). Ductal fluid contains numerous proteins in addition to maspin such as bFGF which may also be a marker for early cancerous changes. These points are made in another of our publications (43). Access to the ductal system of the breast and specifically targeting the myoepithelial-epithelial connection through gene therapy intraductal approaches may prove efficacious in early breast cancer chemoprevention and treatment, strategies claimed in our patent application (44).

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated during the first year of funding that purified myoepithelial maspin does indeed bind to plasma membranes of carcinoma cells via a membrane receptor which in turns inactivates a cellular pathway involved in cellular locomotion.
- We have also found that a number of different pharmacological agents can influence the secretion of maspin from myoepithelial cells and the myoepithelial cells' suppressive phenotype.
- We have demonstrated that antibodies to maspin (polyclonal and monoclonal) are the best discriminator between myoepithelial cells and epithelial cells and they can be used to quantitate the myoepithelial component.
- We have demonstrated that nipple aspirate fluids and ductal lavage fluids obtained by ductoscopy is rich in maspin as a surrogate intermediate end point marker that reflects the integrity of the ductal lobular unit. Ductal fluid contains numerous proteins in addition to maspin such as bFGF which may also be a marker for early cancerous changes. Access to the ductal system of the breast and specifically targeting the myoepithelial-epithelial connection through gene therapy intraductal approaches may prove efficacious in early breast cancer chemoprevention and treatment, strategies claimed in our patent application.

REPORTABLE OUTCOMES

PUBLICATIONS

1. Shao ZM, Radziszewski WJ and **Barsky SH**. Tamoxifen enhances myoepithelial cell suppression of human breast carcinoma progression by two different effector mechanisms. *Cancer Lett*, 157: 133-144, 2000.
2. Nguyen M, Lee MC, Wang JL, Tomlinson JS, Shao ZM and **Barsky SH**. The human myoepithelial cell displays a multifaceted anti-angiogenic phenotype. *Oncogene* 19: 3449-3459, 2000.
3. Alpaugh ML, Lee MC, Nguyen M, Deato M, Dishakjian L and **Barsky SH**. Myoepithelial-specific CD44 shedding contributes to the anti-invasive and anti-angiogenic phenotype of myoepithelial cells. *Experiment Cell Res* 26: 150-158, 2000.
4. Lee MC, Alpaugh ML, Nguyen M, Deato M, Dishakjian L and **Barsky SH**. Myoepithelial specific CD44 shedding is mediated by a putative chymotrypsin-like sheddase. *Biochem Biophys Res Commun* 279: 116-123, 2000.
5. Shen KW, Wu J, Lu JS, Han QX, Shen ZZ, Nguyen M, Shao ZM, and **Barsky SH**. Fiberoptic ductoscopy for patients with nipple discharge. *Cancer* 89: 1512-1519, 2000.
6. Shen KW, Wu J, Lu JS, Han QX, Shen ZZ, Nguyen M, **Barsky SH** and Shao ZM. Fiberoptic ductoscopy for breast cancer patients with nipple discharge. *Surgical Endoscopy*, in press, 2001.
7. Liu YL, Wang JL, Chang H, **Barsky SH**, Nguyen M. Breast cancer diagnosis with nipple fluid bFGF. *Lancet* 356: 567, 2000.

PATENTS AND LICENSES

1. **Barsky SH** and Alpaugh ML. Compositions and methods for intraductal gene therapy. *United States Patent Application*, 60/116,470, filed January 20, 2000.

CONCLUSIONS

1. Mechanism of Maspin Action

Maspin binding to breast carcinoma cells/plasma membranes. The first question that we have addressed is whether maspin exhibits specific, saturable, reversible, and displaceable binding to the surface of breast carcinoma cells in a manner of a ligand-receptor interaction and it does. The mechanism of maspin's effects on invasion and motility inhibition are still unknown. Our studies have shown that in myoepithelial cells it is *secreted* in large amounts. We have been able to purify native maspin to homogeneity. We have obtained *rmaspin* from Dr. Zhang (Baylor). Both *rmaspins* (bacterial and insect) and native maspin derived from myoepithelial cells have been iodinated and incubated with first intact MDA-231, MDA-468, MCF-7, T47D cells and then with their plasma membrane fractions. Excess unlabelled ligand was added and specific binding was determined with Scatchard analysis to calculate the K_d of binding and the # of binding sites/cell or /membrane protein. Specific, displaceable binding indicated a binding protein (receptor). The cell line with the highest maspin binding was used as source to isolate a maspin receptor. In year 02 we will carry this approach further by identification and characterization of a maspin binding protein. Two approaches are envisioned: Maspin will be crosslinked to Sepharose 4B and an affinity column will be made. The plasma membrane preparation from the breast carcinoma cell line exhibiting the highest maspin binding will be extracted, iodinated by the lactoperoxidase method and added to the maspin affinity column. Controls will include BSA cross-linked to Sepharose 4B or cross-linked Sepharose4B alone. The bound fraction will be eluted with 0.2 M glycine HCl pH 3.5, immediately neutralized with 1.0M Tris/saline, lyophilized and run on a gel. Autoradiograms will be used to identify a specific binding protein. Since maspin is a serpin, candidate receptor molecules would include membrane associated proteinases such as the uPA/uPAR complex or MT-MMPs. Since maspin, in addition to inhibiting invasion also inhibits cell motility (which in itself could explain its inhibition of invasion), inhibition by binding to the handful of known motility-stimulating ligand-receptor complexes such as scatter factor/hepatocyte growth factor-c-met receptor, autocrine motility factor-receptor, autoxin-receptor, bFGF and its receptor, interleukin 6 and its receptor, integrins, and E cadherin will be investigated by doing a simultaneous Western blot on the eluted material with antibodies to these different molecules. Since another possibility is that maspin is directly binding a negative regulator of cell motility rather than inhibiting a positive regulator, TGF β -receptor and retinoic acid-receptor complexes, known negative regulators of cell motility will be investigated by Western blotting. If Western blotting reveals no match, affinity labelling will be carried out using iodinated maspin cross-linked to cells with 10 mM disuccinimidyl suberate followed by solubilizing the cells and membranes in reducing sample buffer. The molecular size of the maspin binding protein will be compared to that obtained by maspin affinity chromatography. If these studies show promise of a novel binding protein, it will be purified and sequenced. Recently investigators have shown that maspin binds to single stranded tissue plasminogen activator (ss t-PA); this molecule which is secreted could be a target for maspin action. However in the vast majority of the carcinoma and melanoma lines we studied where maspin exerted a pronounced suppressive effect on both motility and invasion, no ss t-PA was detected in these lines; hence maspin must be acting on a different target. Since both invasion and motility involve complex intracellular pathways, we hypothesize that maspin triggers a signal transduction pathway leading to inhibition of invasion/motility. Instead of a blind search we propose to carry out a directed examination of specific motility genes including ras, and the ras related GTP-binding proteins of the rho subfamily. If maspin results in altered expression of

these genes proof that the binding protein studied is mediating this signal transduction will be determined by competition studies with the solubilized binding protein.

2. Maspin in FNA

We have utilized maspin antibodies on FNA specimens to quantitate the myoepithelial component and determine if this determination discriminates between DCIS and invasive breast carcinoma. Polyclonal (rabbit) and monoclonal (murine) antibodies to maspin have been used (Pharmingen, San Diego, CA) according to standard immunocytochemical cytological protocols. We have great success with this approach and in year 02 will be carrying this approach further.

We shall choose cases known by subsequent biopsy to be either pure DCIS or predominantly invasive carcinoma (most invasive breast cancers have at least some DCIS component). Without knowing the surgical pathology we shall determine first whether there are any myoepithelial cells present on the FNA and we shall determine their number by assessing them by maspin positive immunostaining. We shall determine both the absolute number of myoepithelial cells and the ratio of myoepithelial cells to epithelial cells (the immunopositive to immunonegative ratio) and group the cases where there are no myoepithelial cells and the cases where there are some myoepithelial cells and determine the mean number \pm standard deviation in this second group. We will then subdivide these results into cases of known DCIS and cases of predominantly invasive cancer and do a t test on the results to see if DCIS and invasive breast cancer differ in their average number of myoepithelial cells present on FNA. Analyzing the results in this manner assumes a Gaussian distribution for the number of myoepithelial cells in both groups. The distribution may not however be Gaussian. More likely than not the numbers will be clustered at either end. If this is the case, we will perform a Wilcox non parametric test to tell if the differences in the means of the two groups are significant. If two different and distinct populations do not emerge from this analysis we will analyze the data by logistic regression analysis. Based on the differences in the mean values and/or the slope of this curve we will be able to perform power calculations to determine the number of cases needed to achieve statistical significance. Basically the steeper the slope of this curve the more discriminating the myoepithelial measurement will be and the fewer the number of cases that will be needed. Based also on the differences in the mean values (how many standard deviations apart) of myoepithelial cell numbers or myoepithelial/epithelial ratios between our two groups, DCIS and invasive carcinoma, we will be able also to perform power calculations and determine the number of cases needed. Furthermore we plan to use antibodies to smooth muscle actin, S100 and CALLA, all of which are fairly myoepithelial specific to compare with our maspin results. All of the latter antibodies recognize structural myoepithelial components and as such quantitate myoepithelial cell number. Maspin, on the other hand, not only identifies myoepithelial cell number but, if our hypothesis is correct, provides an index of myoepithelial cell function---after all maspin is a serpin and a paracrine tumor suppressor. Therefore the use of maspin in this setting may be more informative.

3. Maspin in Ductal Fluid

Maspin in ductal fluid was measured by Western blot with total protein normalization. Human subjects approval for the collection of ductal fluid through select duct cannulation was obtained.. Small aliquots of this collected ductal fluid (50 patients) was used for the present studies. Nipple aspirates were collected by nipple suction (Sartorius/Petrakis). Analysis of nipple aspirates

revealed the pooled contributions of all or most of the ducts where selective ductal cannulation allowed for a discrimination of one duct from another (eg, a duct with DCIS or microcalcifications v a normal duct. Our findings indicated that maspin was present reflecting the integrity of the ductal-lobular unit. We also demonstrated maspin in saliva, a fluid which reflects the abundance of myoepithelial cells in the salivary glands.

In year 02, ductal fluid will be collected following cannulation and washing of selected ducts in patients with microcalcifications on screening mammography who are about to undergo either excisional or core biopsy. Paired comparisons of maspin levels in ductal fluid obtained from ducts harboring microcalcifications or DCIS and normal ducts from the same patients will be made. Maspin levels will be correlated with the histopathology surrounding the microcalcifications. It is anticipated that some of these patients will exhibit normal ductal histopathology surrounding their microcalcifications, some will harbor proliferations like hyperplasia, adenosis, ADH, and DCIS and still others invasive carcinoma. The screening value of maspin levels in all of these patients will be determined. Our approach to the evaluation of maspin values and to their discriminatory ability among the different groups will be similar to that given above for the analysis of myoepithelial cell number. Measurements of *myoepithelial* maspin in ductal fluid will be compared to levels of a breast *epithelial* cell marker such as carcinoembryonic antigen (CEA) or bFGF. We, in fact, recently found that bFGF is elevated in ductal fluid of certain patients. Hence the maspin/CEA or maspin/bFGF ratio might be predictive of risk with increased maspin/CEA or increased maspin/bFGF correlating with normalcy and decreased maspin/CEA or decreased maspin/bFGF correlating with either high risk, microcalcifications and/or precancerous histopathology.

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APPENDICES

Tamoxifen enhances myoepithelial cell suppression of human breast carcinoma progression in vitro by two different effector mechanisms

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Abstract

Our previous studies have indicated that myoepithelial cells surrounding ductal and acinar epithelium of glandular organs, such as the breast, exert multiple paracrine suppressive effects on incipient and developing cancers that arise from this epithelium. Myoepithelial cells and derived cell lines (HMS 1–6) exert these effects through the secretion of a number of different effector molecules that exert anti-invasive, anti-proliferative, and anti-angiogenic activities. Since previous basic and clinical studies have examined the role of estrogen agonists and antagonists on human breast cancer cells and because issues of hormone replacement therapy (HRT) and tamoxifen chemoprevention are such timely issues in breast cancer, we wondered whether or not hormonal manipulations might affect myoepithelial cells in vitro as far as their paracrine suppressive activities on breast cancer were concerned. The present in vitro study demonstrates that treatment of myoepithelial cells with tamoxifen but not 17 β -estradiol increases both maspin secretion and invasion-blocking ability. Furthermore tamoxifen but not 17 β -estradiol increases inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production by myoepithelial cells when they are co-cultured with conditioned media from or breast carcinoma cells directly. This increased myoepithelial NO exerts both autocrine and paracrine antiproliferative effects which can be blocked by inhibition of iNOS. 17 β -Estradiol, however, competes with all of these suppressive effects of tamoxifen suggesting that the mechanism of tamoxifen action is estrogen receptor mediated. Myoepithelial cells lack ER- α but express ER- β . Tamoxifen, but not 17 β -estradiol, increases AP-1 CAT but not ERE-CAT activity. Again, 17 β -estradiol competes with the transcription-activating effects of tamoxifen. These experiments collectively suggest that the actions of tamoxifen on the increased secretion of maspin and increased production of NO by myoepithelial cells are mediated through ER- β and the transcription-activation of an ER-dependent AP-1 response element. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Myoepithelial cell; ER- β ; Tumor suppression; Tamoxifen; Maspin; Nitric oxide

1. Introduction

Our previous studies have indicated that myoe-

epithelial cells surrounding ductal epithelium of glandular organs such as the breast exert multiple paracrine suppressive effects on incipient cancers that arise from this epithelium [1–3]. This paracrine suppression may keep the genetic alterations occurring within malignant epithelial cells in check so that the evolving cancer exists for a number of years only as an in situ

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lesion confined within the ductal system [4]. This in situ lesion is commonly termed ductal carcinoma in situ or DCIS. Due to their close proximity, myoepithelial cells would be anticipated to exert important paracrine influences on both normal, precancerous, and cancerous epithelial cells. Myoepithelial cells of the breast differ from luminal ductal and acinar epithelial cells in many ways: they lie next to the basement membrane and contribute to the synthesis of that structure; they rarely transform or proliferate and when they do give rise to only low-grade benign neoplasms [5,6]. Myoepithelial cells, in a sense, can be regarded then as both autocrine as well as paracrine tumor suppressors. Our laboratory has established immortalized myoepithelial cell lines and transplantable xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3), breast (HMS-4, HMS-5) and bronchus (HMS-6) (with their respective xenografts designated as HMS-#X) [1–3,5,6]. These cell lines and xenografts express identical myoepithelial markers as normal myoepithelial cells in situ and display an essentially normal diploid karyotype. In previous studies we have demonstrated that our myoepithelial cell lines/xenografts and myoepithelial cells in situ constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α -1 anti-trypsin, an unidentified 31–33 kDa trypsin inhibitor, thrombospondin-1, soluble bFGF receptors, and maspin [1–3]. Our human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion [1,3] and endothelial cell migration and proliferation (angiogenesis) in vitro [7]. Our myoepithelial cell lines also inhibit breast carcinoma proliferation in vitro through an induction of breast carcinoma cell G₂/M arrest and apoptosis [3], the latter phenomenon of which also occurs in situ within DCIS [8]. On the basis of our immunoprecipitation studies, secreted myoepithelial cell maspin is a major effector molecule that inhibits invasion but not proliferation [3]. Candidate myoepithelial molecules that mediate antiproliferative and antiangiogenic effects on carcinoma cells and endothelial cells respectively include nitric oxide (NO) and thrombospondin-1 [4].

The effects of hormone replacement therapy (HRT) and tamoxifen chemoprevention are timely issues for patients at risk for developing breast cancer but

previous basic and clinical studies examining the effects of these hormones in human breast cancer have focused mainly on their direct actions on carcinoma cells themselves. For this reason, we wondered whether these hormones could conceivably affect breast carcinoma cells indirectly by their actions on myoepithelial cells. In the present study we specifically examined the hormonal regulation of the tumor suppressor phenotype of myoepithelial cells in vitro by focusing on two myoepithelial-associated effector molecules: maspin and NO.

2. Materials and methods

2.1. Reagents

Tamoxifen, 17 β -estradiol, PMA (phorbol 12-myristate 13-acetate), dexamethasone, and hydrocortisone were purchased from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium (MEM), keratinocyte serum-free medium (KSFM) and fetal bovine serum (FBS) were obtained from Life Technologies Inc./Gibco (Grand Island, NY). [α -³²P]dCTP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

2.2. Cell lines

The ER-positive (MCF-7 and T-47D) and ER-negative (MDA-MB-231 and MDA-MB-468) cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, MD). MCF-7, T-47D, MDA-MB-231, and MDA-MB-468 cells were maintained in Eagle MEM with 10% FBS and antibiotics (50 units/ml penicillin and 50 μ g/ml streptomycin). Human myoepithelial cell lines (HMS-1, HMS-3, HMS-4, HMS-5, and HMS-6), established in our laboratory [1–3,5,6], were maintained in KSFM with epidermal growth factor and bovine pituitary extract supplements. All cells were cultured as monolayers in 95% air/5% CO₂. Conditioned media (CM) was prepared and concentrated as previous described [1].

2.3. In vitro assays of invasion

Invasion experiments were conducted with an invasion chamber utilizing Matrigel from Collaborative

Biomedical Products (Bedford, MA) as described in previous studies [1], using highly invasive breast carcinoma cell lines. The Matrigel invasion experiments were carried out according to standard protocols using an intervening layer of HMS-1 cells or concentrated CM from HMS-1 cells [1,9]. Our previous studies had shown that CM from HMS-1 cells had to be concentrated at least 25-fold for invasion-inhibitory activity to be observed [1]. Treatment of HMS-1 cells was carried out for 24 h with the following agents: PMA (5 μ M), dexamethasone (0.25 μ M), tamoxifen (10^{-8} , 10^{-7} , or 10^{-6} M) and 17 β -estradiol (10^{-8} , 10^{-7} , or 10^{-6} M). For competition experiments, the dose of 17 β -estradiol was increased to 10^{-5} M. Other myoepithelial cell lines were studied in similar fashion.

2.4. Growth experiments

HMS-1 cells were plated in 24-well plates at an initial cell concentration of 1×10^4 cells per well in supplemented KSM, treated with various concentrations and combinations of tamoxifen and 17 β -estradiol, and counted after 3, 6, and 9 days. Other myoepithelial cell lines were studied in similar fashion. The cells were also assayed for apoptosis by the ApopTag detection system (Oncor, Gaithersburg, MD) as performed in previous studies [3]. The effects of inhibition of inducible nitric oxide synthase (iNOS) by hydrocortisone (5 μ M) were also studied in these settings.

2.5. Northern, Western, and immunoprecipitation studies

Total RNA was extracted, 20 μ g of total RNA was loaded per lane in 1.2% agarose gels, and Northern blot analyses were performed as previously described [1]. The full-length human ER- α cDNA probe was provided Dr P. Chambon (IGBMC, France); the full-length human iNOS cDNA probe was provided by Dr Louis Ignarro (UCLA, Los Angeles, CA); a maspin cDNA probe was provided by Dr Ruth Sager (Dana-Farber Cancer Institute, Boston, MA). Probes were labeled according to the random primer method of Feinberg and Vogelstein [10]. Normalization was achieved with a 36B4 cDNA probe (ATCC). Scion Image software was used for densitometric analysis of the bands. Western blots were performed as previously described [1] using a mouse monoclonal

antibody to maspin (PharMingen, San Diego, CA) with the manufacturer's recommended dilutions and a 1:50 000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL) followed by development of the reaction with the ECL detection system (Amersham Life Sciences, Arlington Heights, IL).

Concentrated HMS-1 CM (25 \times) was immunoprecipitated by using anti-maspin antibody (PharMingen) at various dilutions (1:100–1:2000) followed by immunobeads (Bio-Rad). Following centrifugation, the supernatants were harvested, filtered through a 0.2- μ m filter, and saved for invasion experiments and Western analysis. The immunoprecipitates were analyzed by Western blot. Control antibodies used included mouse monoclonals anti-human PAI-1 and PAI-2 (American Diagnostica, Greenwich, CT).

2.6. Determination of nitric oxide (NO) and iNOS expression

HMS-1 cells were cultured both in the presence and absence of MDA-MB-231 cells and in the presence and absence of MDA-MB-231 CM (25 \times). The cells received either added tamoxifen (10^{-7} M), 17 β -estradiol (10^{-7} M) or various concentrations and combinations. After various time periods (0–24 h), the HMS-1 cells in the CM experiments were harvested for Northern blot analysis. In both the co-culture experiments and the CM experiments, the supernatants were analyzed after 24–72 h for nitrite content. NO, quantified by the accumulation of nitrite (as a stable end product) in the supernatants, was determined by a microplate assay method [11]. Briefly, 100 μ l samples were removed from the conditioned supernatants and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-1-naphthylethylene diamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve and expressed in micromolar amounts. Values were normalized for total cellular protein and NO expressed in arbitrary units. In the co-culture experiments, the cells were also assayed for apoptosis by the ApopTag detection system (Oncor, Gaithersburg, MD) as performed in previous studies [3]. The effects

of inhibition of iNOS by hydrocortisone (5 μ M) were also studied in these settings.

2.7. Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA (1 μ g per reaction), denatured at 65°C for 5 min, was reverse transcribed in a final volume of 10 μ l containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 μ M random hexamers (Gibco/BRL) and 150 units M-MLV reverse transcriptase (Gibco/BRL). The reaction was allowed to proceed for 60 min at 37°C and then terminated by heating at 90°C for 5 min. One microliter of this reaction was amplified by PCR in a final volume of 10 μ l containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTPs (dATP, dTTP, dGTP and [³²P]dCTP), 4 ng/ μ l of each primer, and 0.02 unit/ μ l of Taq DNA polymerase (Gibco/BRL). The primers used to amplify ER- β cDNA were: sense, 5'-TGCTTTGGTTTGGGTGATTGC-3' (nucleotides 1164–1185); antisense, 5'-TTTGCTTTTACTGTC-

CTCTGC-3' (nucleotides 1402–1423) [12]. The PCR conditions were 1 min 94°C, 30 s 58°C, and 30 s 72°C, for 30 cycles. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel using unlabeled dNTPs.

2.8. CAT assay

Since the action of estrogen agonists/antagonists bound to estrogen receptors (either ER- α or ER- β) activate downstream genes containing either a classical ERE or an ER-dependent AP-1 response element, myoepithelial cell lines were transfected by a Lipofectamin method (Gibco/BRL) with CAT-reporter constructs fused to heterologous promoters containing the human estrogen response element (ERE-tk-CAT) (provided by Dr P. Chambon, IGBMC, France) or AP-1-tk-CAT (provided by Dr Ronald Evans, Howard Hughes Medical Institute, La Jolla, CA). Transient transfections were performed in the presence of 4 μ g of a β -galactosidase expression vector to correct for differences in transfection efficiencies. After trans-

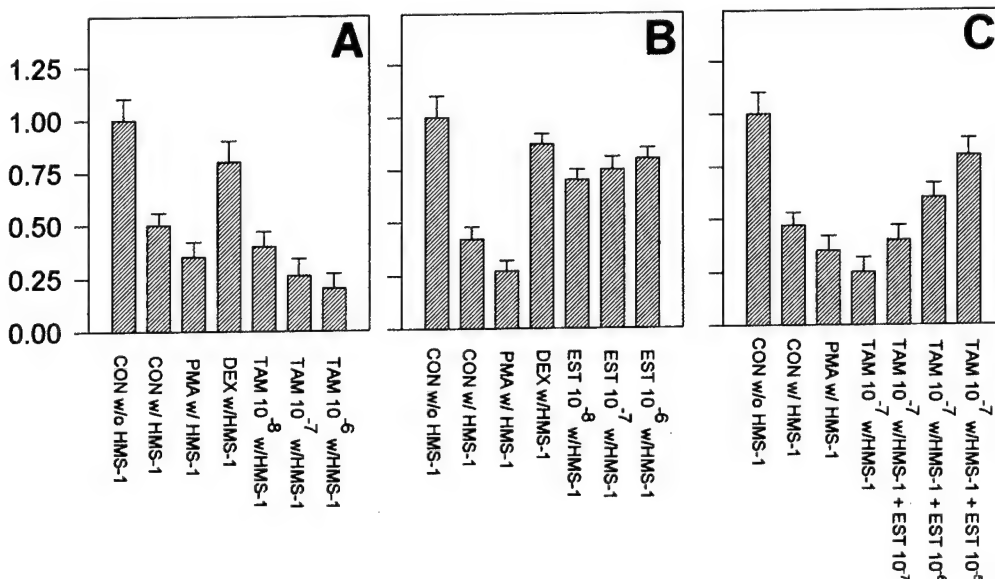


Fig. 1. Treatment of HMS-1 cell with estrogen antagonists/agonists at various concentrations of tamoxifen (A), 17 β -estradiol (B), and various combinations (C) are compared with anti-invasive potentiating effects of PMA and anti-invasive abolishing effects of dexamethasone. The most highly invasive breast carcinoma cell line, MDA-MB-231, is depicted. Constitutive levels of invasion of these cells in the absence of intervening HMS-1 cells (CON without HMS-1) is assigned an arbitrary value of 1.0. The effects of HMS-1 cells (CON with HMS-1) and HMS-1 cells pretreated with various agents is expressed relative to this control level. The data represent mean \pm SEM of three independent experiments. Results using other breast carcinoma cell lines and other myoepithelial cell lines were similar to results depicted for the MDA-MB-231 and HMS-1 cell lines, respectively.

fection, the cells were treated with 10^{-7} M tamoxifen, 10^{-7} M 17β -estradiol, or various concentrations and combinations, and harvested at the end of a 72-h incubation period. CAT activity was measured by thin layer chromatography by incubating the cell lysate at 37°C for 2 h with $4\text{ }\mu\text{M}$ [^{14}C]chloramphenicol and 1 mg/ml acetyl coenzyme A. Acetylated [^{14}C]chloramphenicol was quantitated.

2.9. Statistical analysis

Results were analyzed with standard tests of statistical significance, including the two-tailed Student's *t*-test and a one-way analysis of variance (ANOVA).

3. Results

3.1. Hormonal modulation of the anti-invasive phenotype of myoepithelial cells

HMS-1 and its CM (25 \times) exerted broad anti-invasive activity against both ER-positive (MCF-7 and T47D) and ER-negative (MDA-MB-231 and MDA-MB-468) lines. As reported previously, treatment of HMS-1 cells with different pharmacological agents, phorbol 12-myristate 13-acetate (PMA) ($5\text{ }\mu\text{M}$) and dexamethasone ($0.25\text{ }\mu\text{M}$) for 24 h had opposite effects on the anti-invasive phenotype. Dexamethasone nearly abolished the anti-invasive phenotype whereas PMA potentiated it. The effects of tamoxifen were similar to the anti-invasive potentiating effects of PMA (Fig. 1A). The anti-invasive potentiating effects of tamoxifen were dose-dependent (Fig. 1A). In contrast, 17β -estradiol reduced the anti-invasive effects of myoepithelial cells (Fig. 1B). Competition experiments between tamoxifen and 17β -estradiol showed that increasing concentrations of 17β -estradiol blocked the anti-invasive potentiating effects of tamoxifen (Fig. 1C).

3.2. Hormonal modulation of myoepithelial cell maspin and its anti-invasive effects

Immunoprecipitation of maspin from HMS-1 CM (Fig. 2A) reversed the anti-invasive effects of myoepithelial CM on breast carcinoma cell invasion in vitro (Fig. 2B). Tamoxifen treatment of HMS-1 resulted in a 2–3-fold increase in maspin secretion

with increasing doses of tamoxifen (Fig. 3A) and increasing times of exposure (Fig. 3B). 17β -Estradiol, in contrast, exerted no effects on maspin secretion (Fig. 3C) and completely abolished the maspin stimulatory effects of tamoxifen in competition experiments (Fig. 3D). Tamoxifen's increase in maspin secretion was not due to an increase in steady state maspin mRNA levels which were essentially unchanged by this treatment (Fig. 3E).

3.3. Hormonal modulation of myoepithelial cell proliferation

Increasing and prolonged tamoxifen exposure resulted in striking anti-proliferative effects on myoepithelial cells (Fig. 4A) whereas increasing and prolonged 17β -estradiol exposure exerted no such

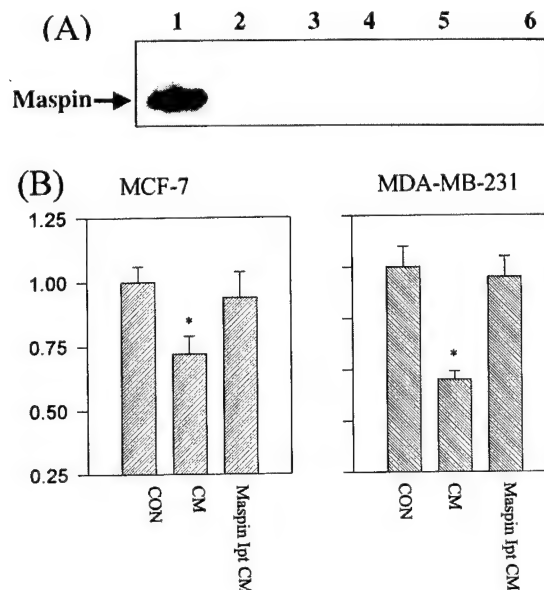


Fig. 2. (A) Maspin immunoprecipitation fraction at various dilutions of maspin antibody. Lane 1, 1/100; lane 2, 1/500; lane 3, 1/1000; lane 4, 1/2000. Optimal dilution was 1/100 to achieve nearly 100% immunoprecipitation. Other serpin antibodies used including anti-PAI-1 (lane 5) and anti-PAI-2 (lane 6) resulted in only negligible cross-reacting immunoprecipitation of maspin. (B) Effects of HMS-1 25 \times CM and maspin-immunoprecipitated CM on breast carcinoma invasion. Control levels of invasion of designated breast carcinoma cell lines, MCF-7 and MDA-MB-231 were assigned arbitrary values of 1.0 and effects of CM and immunoprecipitated CM were expressed relative to these control levels. Results with other myoepithelial cell lines were similar. *Statistically significant differences compared with control ($P < 0.05$).

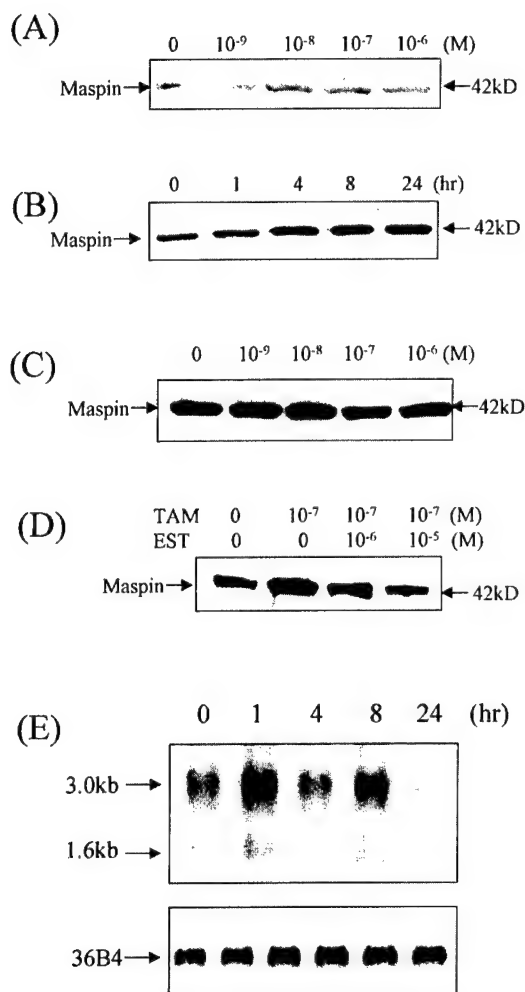


Fig. 3. (A) Dose response of tamoxifen (10⁻⁹–10⁻⁶ M) treatment on maspin secretion over 24 h measured by Western blot with anti-maspin of HMS-1 CM (25 ×). (B) Effects of tamoxifen (10⁻⁷ M) treatment on maspin secretion for various times (h) of tamoxifen exposure. (C) Dose response of 17β-estradiol (10⁻⁹–10⁻⁶ M) on maspin secretion by HMS-1 cells over 24 h. 17β-Estradiol exerted no stimulatory effects. (D) In competition experiments, increasing concentrations of 17β-estradiol completely blocked tamoxifen's stimulation of maspin secretion. (E) Northern blot of maspin steady-state mRNA levels following exposure of HMS-1 cells to tamoxifen (10⁻⁷ M) for indicated time periods. Two maspin transcripts (3.0, 1.6 kb) are present but no stimulatory effects of tamoxifen on maspin steady-state mRNA levels are seen. At 24 h, a slight decrease in steady-state mRNA levels was observed. Normalization was with 36B4. Results with other myoepithelial cell lines were similar.

effects (Fig. 4B) and blocked the anti-proliferative effects of tamoxifen in competition experiments (Fig. 4C). In the tamoxifen experiments only, a 3-fold increase in apoptotic index was observed; however, when the tamoxifen experiments were conducted in the presence of hydrocortisone (5 μM) to inhibit iNOS, no anti-proliferative effects and no increase in apoptotic index were observed (data not shown).

3.4. Hormonal modulation of myoepithelial cell NO and iNOS expression

Breast carcinoma CM (25 ×) was synergistic with tamoxifen but not 17β-estradiol in increasing myoepithelial cell NO production (Fig. 5A); this synergism was even more striking in co-culture experiments of MDA-MB-231 cells with HMS-1 cells (Fig. 5B). In both situations, 17β-estradiol blocked tamoxifen's synergistic effect on increased NO production (Fig. 5A,B). Tamoxifen together with MDA-MB-231 CM (25 ×) increased steady state iNOS mRNA levels over time (Fig. 5C,D). This effect was not observed with 17β-estradiol and was blocked by 17β-estradiol in competition experiments (data not shown). In the co-culture experiments with tamoxifen, an increase in apoptotic index was observed in both cell populations; however, this increase in apoptosis was blocked by the inhibition of iNOS and NO with the addition of hydrocortisone (5 μM) (data not shown).

3.5. Hormonal modulation of the myoepithelial cell's suppressor phenotype through ER-β

Myoepithelial cell lines lacked ER-α expression (Fig. 6A) but uniformly expressed ER-β (Fig. 6B). Since the action of estrogen agonists/antagonists bound to estrogen receptors (either ER-α or ER-β) activate downstream genes containing either a classical ERE or an ER-dependent AP-1 response element, myoepithelial cell lines were transfected with CAT-reporter constructs fused to heterologous promoters containing the human estrogen response element (ERE-tk-CAT) or AP-1-tk-CAT. Tamoxifen (10⁻⁷ M) increased AP-1-CAT activity 3-fold (Fig. 7A,B). This effect was not observed with 17β-estradiol. Furthermore 17β-estradiol (10⁻⁵ M) competed with and blocked the effects of tamoxifen (10⁻⁷ M) (Fig. 7A,B). 17β-Estradiol (10⁻⁷ M) did increase ERE-

CAT activity but tamoxifen (10^{-7} M) did not (Fig. 7C,D).

4. Discussion

Cancer cells come under the influence of important paracrine regulation from the host microenvironment [13]. Such host regulation may be as great a determinant of a tumor cell's behavior in vivo as the specific oncogenic or suppressor alterations occurring within the malignant cell itself and may be mediated by specific extracellular matrix molecules, matrix-associated growth factors, or host cells themselves [14,15]. Both positive (fibroblast, myofibroblast and endothelial cell) and negative (tumor infiltrating lymphocytes, and cytotoxic macrophages) cellular regulators exist that profoundly affect tumor cell behavior in vivo [16,17]. One host cell, the myoepithelial cell, appears to belong to the negative cellular regulator group [1]. Our previous studies have shown that myoepithelial cells and derived cell lines exert suppressive effects on breast carcinoma cells through secretion of a number of different anti-invasive [1], anti-proliferative [3], and anti-angiogenic molecules [7]. Since issues of hormone replacement therapy and tamoxifen chemoprevention are timely, we wondered whether or not hormonal manipulations might affect myoepithelial cells directly in so far as

their paracrine suppressive activities on breast cancer were concerned. In this study we specifically focused on two such myoepithelial-associated suppressor molecules: maspin and NO.

Treatment of myoepithelial cells with tamoxifen resulted in 2–3-fold increase of maspin, a recently identified tumor suppressor [18–21]. It had been shown in transfection studies that maspin was capable of inhibiting breast carcinoma cell growth, invasion, and metastasis in an autocrine manner [18–21]. Recently, it had also been demonstrated that rMaspin reduced the invasive phenotype of MDA-MB-435 cells by altering their integrin profile, particularly $\alpha 5$, which in turn converted these cells to a more benign epithelial phenotype, with less invasive ability [22]. We have recently demonstrated that maspin suppresses invasion in a paracrine manner [1]. Pharmacological enhancement of maspin secretion should therefore enhance tumor suppressor activity. In our study the anti-invasive effects of maspin were in evidence in both co-culture experiments and in CM experiments. In the co-culture experiments the invasive cells were placed in an upper chamber and an intervening layer of myoepithelial cells were placed in underlying Matrigel as previously reported [1]. Since myoepithelial cells were non-invasive, reversing the polarity of this system did not yield meaningful data. In the CM experiments $25 \times \text{CM}$ was selected because in our previous studies [1], CM

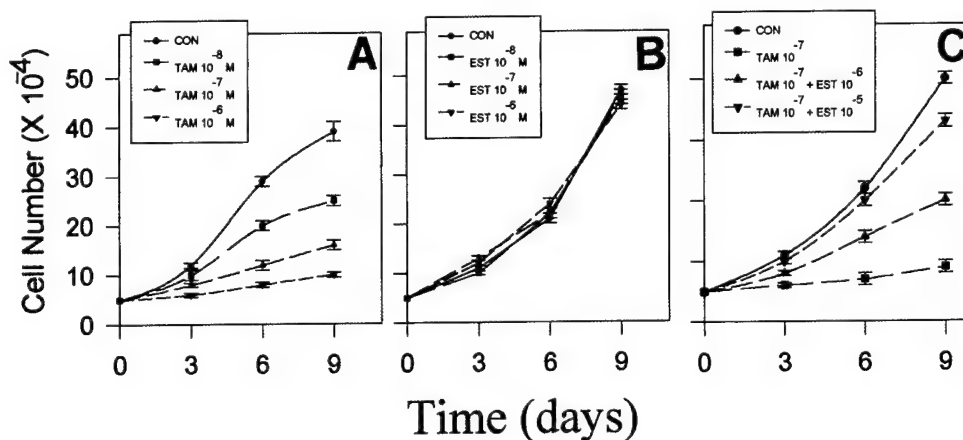


Fig. 4. Effects at various doses of tamoxifen (A), 17β-estradiol (B), and various concentrations and combinations (C) on HMS-1 cell proliferation. Tamoxifen inhibited proliferation, 17β-estradiol had no effect, and 17β-estradiol blocked tamoxifen's antiproliferative effects in competition experiments. The data represent mean \pm SEM of three independent experiments. Results with other myoepithelial cell lines were similar.

had to be concentrated at least 25–100× for invasion-inhibitory activity to be observed. Our measurements of maspin in the co-culture system were approxi-

mately those of unconcentrated CM. The reason why the co-culture system with its intervening layer of myoepithelial cells was effective at inhibiting inva-

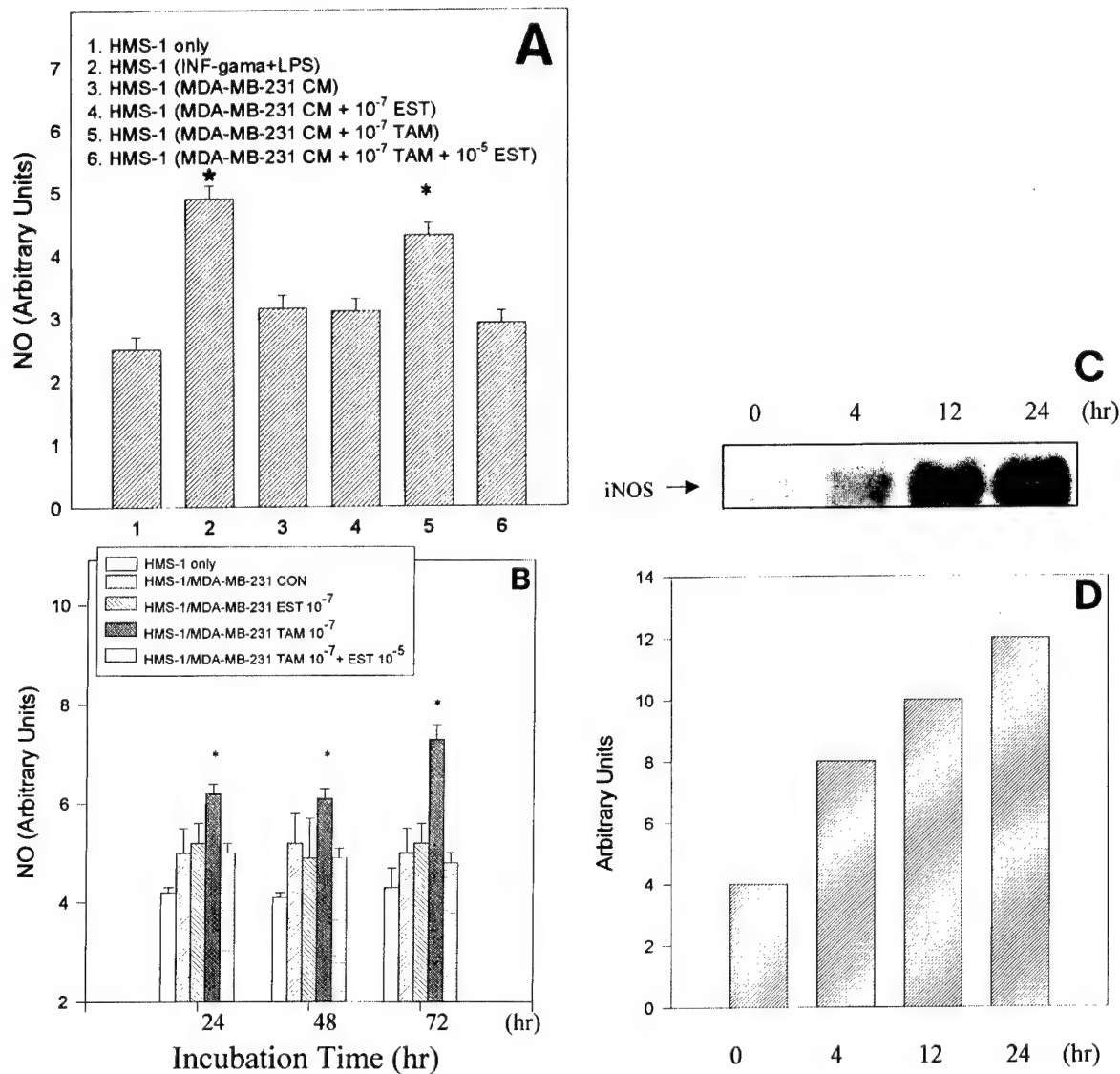


Fig. 5. (A) Nitric oxide (NO) activity in HMS-1 cells with various additions treated for 72 h. Lane 1, HMS-1 only; lane 2, HMS-1 incubated with the cytokine inducers INF- γ and LPS; lane 3, HMS-1 cells incubated with MDA-MB-231 CM (25 \times); lane 4, HMS-1 cells incubated with MDA-MB-231 CM (25 \times) plus 10^{-7} M 17 β -estradiol; lane 5, HMS-1 cells incubated with MDA-MB-231 CM (25 \times) plus 10^{-7} M tamoxifen; lane 6, HMS-1 cells incubated with MDA-MB-231 CM (25 \times) plus 10^{-7} M tamoxifen and 10^{-5} M 17 β -estradiol. *Statistically significant differences compared with control ($P < 0.05$). (B) NO activity in HMS-1 cells co-cultured with MDA-MB-231 cells treated with various additions for indicated times. Tamoxifen, but not 17 β -estradiol, was effective at increasing NO production. 17 β -Estradiol, furthermore, blocked tamoxifen's effect of increasing NO production. *Statistically significant differences compared with control. (C) iNOS expression by Northern blot in HMS-1 cells treated with MDA-MB-231 CM (25 \times) plus 10^{-7} M tamoxifen for increasing time periods (0–24 h). Results with other myoepithelial cell lines were similar. (D) Densitometric analysis of bands of iNOS expression by Northern blot.

sion is probably because the myoepithelial cells provide a gradient of maspin that the invading tumor cells encounter as they approach. So, in the co-culture system, the concentration of maspin in the invading tumor cell's microenvironment, may, in fact, be much greater than in the 25 × CM experiments.

NO is derived from L-arginine by constitutive nitric oxide synthase (NOS) or inducible nitric oxide synthase (iNOS). NO produces multiple suppressive effects on cancer cells. Recently, it has been shown that the production of endogenous NO is associated with apoptosis of tumor cells, suppression of tumorigenicity, and the abrogation of metastasis [23,24]. In the present study, we have demonstrated that iNOS is present in myoepithelial cells and both iNOS expression and NO production is increased by tamoxifen in the presence of breast carcinoma cell CM. Tamoxifen also exerts antiproliferative effects on myoepithelial cells. These findings implicate NO as another important suppressor molecule made by myoepithelial cells

that is hormonally regulated. To confirm these observations we have provided a functional assay to demonstrate NO action. We have conducted additional experiments using hydrocortisone (5 μ M), an inhibitor of iNOS. We have demonstrated that in this setting the anti-proliferative effects of tamoxifen on myoepithelial cells are not observed. In addition in the co-culture experiments where the MDA-MB-231 line was mixed with HMS-1 cells, in the presence of tamoxifen we noted increased apoptosis after 72 h in both populations of cells. However, in the presence of hydrocortisone (5 μ M), this increased apoptosis was not observed. In both the HMS-1 growth experiments and in the co-culture experiments, the inhibition of iNOS by hydrocortisone did not affect maspin levels. Although these studies are preliminary, they suggest that the increased NO released by myoepithelial cells in response to tamoxifen has functional significance in terms of anti-proliferative and apoptotic tumor suppressive effects.

Estrogen agonists and antagonists can exert effects on their cellular targets through a number of mechanisms: some of these are rapid direct membrane effects of the hormone on ion channels [25] or on the induction of susceptibility to oxidative stresses that do not require receptor binding and downstream transcriptional activation of genes. Other effects of estrogen agonist and antagonist action are mediated through receptor binding, either ER- α or ER- β . Our myoepithelial cells completely lacked expression of ER- α but expressed ER- β . Estrogen action was initially thought to mediated principally through a single ER, ER- α , a member of the steroid/thyroid/retinoic acid receptor superfamily [26]. Recently, however, a second ER, ER- β was identified in the rat, mouse, and human [27,28]. ER- β shares a similar structural and functional composition with ER- α and has been shown to activate the transcription of similar ERE-containing target genes [27,28]. However 17 β -estradiol and tamoxifen have been shown to differentially activate other ER downstream genes containing ER-dependent AP-1 response elements [29]. In our study it was important to distinguish which mode of action was likely responsible for tamoxifen's enhancement of the anti-invasive phenotype of myoepithelial cells and the enhanced secretion of maspin and production of NO. The fact that 17 β -estradiol competed with tamoxifen in blocking all of its actions on myoepithe-

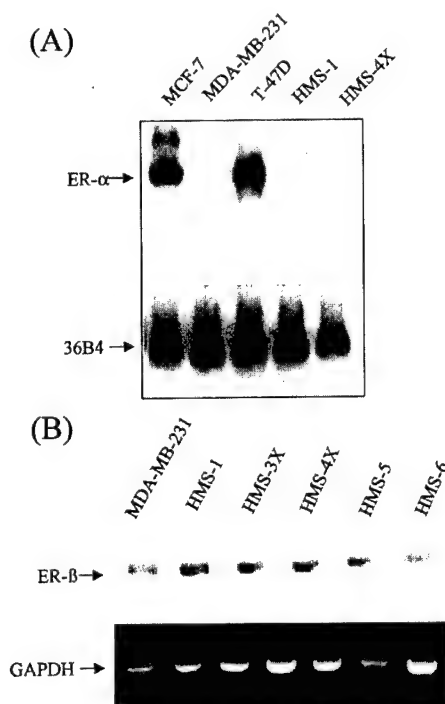


Fig. 6. Expression of ER- α and ER- β in representative myoepithelial cell lines. (A) Northern blot analysis, ER- α expression. Normalization was with 36B4. (B) ER- β expression by RT-PCR. GAPDH served as a housekeeping control.

lial cells including its anti-invasive potentiating effects, its stimulation of effector molecules: maspin and NO, and its transcription-activation of an ER-dependent AP-1 response element supports that tamoxifen's mode of action on myoepithelial cells is through the estrogen receptor, in this case, ER- β . In our study tamoxifen, but not 17 β -estradiol upregulated AP-1-CAT activity but not ERE-CAT activity. These findings suggest that the downstream events of iNOS expression, NO production, and maspin secretion are mediated through tamoxifen's binding to ER- β and subsequent ER- β -AP-1, rather than ER- β -ERE, *trans*-activation.

The effects of HRT and tamoxifen chemoprevention are timely issues for patients at risk for developing breast cancer. It must be emphasized that all of

these exogenous hormones exert multiple effects on multiple organ systems, some desired, some untoward that have global implications [30,31]. It is likely that not all of the effects of these hormones have been defined. The present study has examined the effects of tamoxifen and 17 β -estradiol on myoepithelial cells in vitro. It is interesting that whereas tamoxifen increased the anti-invasive and tumor-suppressive effects of myoepithelial cells by maspin and NO-dependent mechanisms, 17 β -estradiol reduced the anti-invasive effects of myoepithelial cells by maspin and NO-independent mechanisms. We can only speculate at this point concerning these latter mechanisms. In our previous study [1], we noted that dexamethasone similarly reduced the anti-invasive effects of myoepithelial cells by a maspin-independent mechanism.

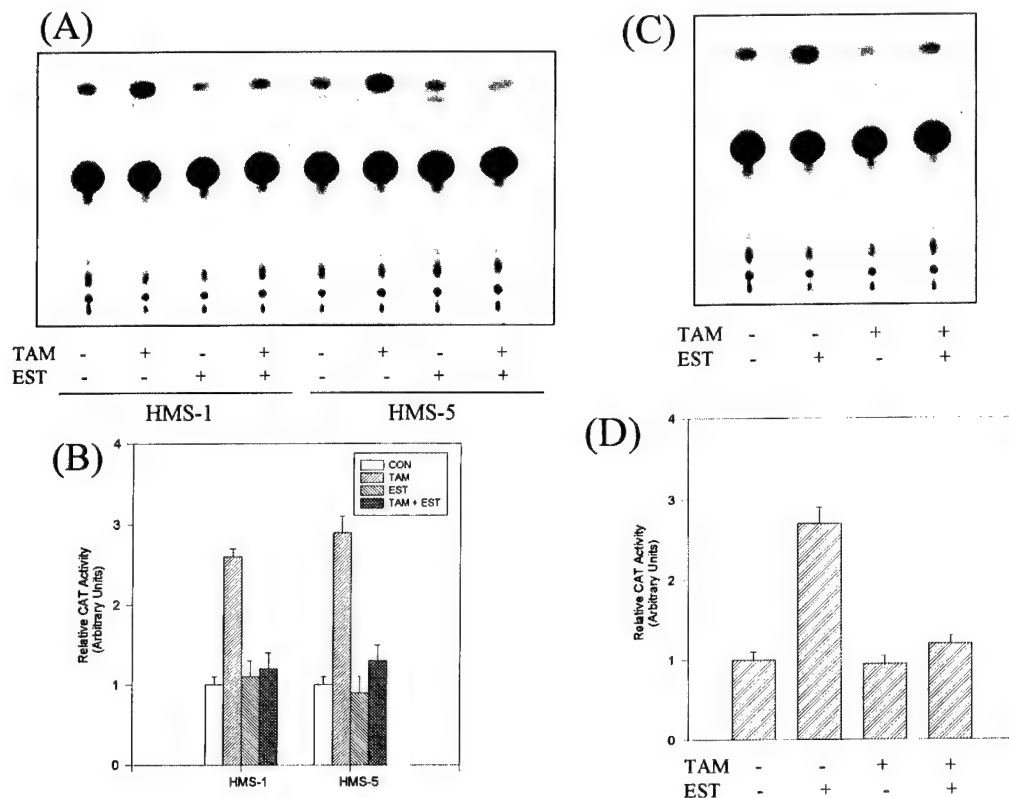


Fig. 7. (A) Tamoxifen (10^{-7} M) stimulation of AP-1-CAT activity in representative myoepithelial cell lines, HMS-1 and HMS-5. 17 β -Estradiol (10^{-7} M) exerted no such stimulatory effects and blocked the effects of tamoxifen at high doses (10^{-5} M). (B) Results depicted are the means of three independent experiments. Error bars represent standard errors. (C) Tamoxifen (10^{-7} M) exerted no stimulation of ERE-CAT activity in HMS-1 whereas 17 β -estradiol (10^{-7} M) stimulated a 2–3-fold increase in ERE-CAT which was blocked by tamoxifen (10^{-5} M). (D) Results depicted are the means of three independent experiments. Error bars represent standard errors.

ism. So possibly 17 β -estradiol and dexamethasone act similarly. It should be pointed out that in our hormonal studies with 17 β -estradiol, although we used amounts comparable to the amounts used in numerous other in vitro studies, the amounts used (10^{-8} – 10^{-6} M) reflect non-physiological doses. Their relevance to HRT issues can therefore be questioned as the level of active hormones in HRT would not be expected to get beyond an equivalence of 10^{-9} M. The tamoxifen amounts (10^{-8} – 10^{-6} M) we used in our in vitro experiments, in contrast, were not above what is found in patients [32] and our observations with tamoxifen may more likely have clinical relevance.

Acknowledgements

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The human myoepithelial cell displays a multifaceted anti-angiogenic phenotype

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Human myoepithelial cells which surround ducts and acini of certain organs such as the breast form a natural border separating epithelial cells from stromal angiogenesis. Myoepithelial cell lines (HMS-1-6), derived from diverse benign myoepithelial tumors, all constitutively express high levels of active angiogenic inhibitors which include TIMP-1, thrombospondin-1 and soluble bFGF receptors but very low levels of angiogenic factors. These myoepithelial cell lines inhibit endothelial cell chemotaxis and proliferation. These myoepithelial cell lines sense hypoxia, respond to low O₂ tension by increased HIF-1 α but with only a minimal increase in VEGF and iNOS steady state mRNA levels. Their corresponding xenografts (HMS-X-6X) grow very slowly compared to their non-myoepithelial carcinomatous counterparts and accumulate an abundant extracellular matrix devoid of angiogenesis but containing bound angiogenic inhibitors. These myoepithelial xenografts exhibit only minimal hypoxia but extensive necrosis in comparison to their non-myoepithelial xenograft counterparts. These former xenografts inhibit local and systemic tumor-induced angiogenesis and metastasis presumably from their matrix-bound and released circulating angiogenic inhibitors. These observations collectively support the hypothesis that the human myoepithelial cell (even when transformed) is a natural suppressor of angiogenesis. *Oncogene* (2000) 19, 3449–3459.

Keywords: thrombospondin-1; hypoxia inducible factor-1; angiogenic inhibitors; angiogenic factors

Introduction

Cancer cells come under the influence of important paracrine regulation from the host microenvironment (Cavenee, 1993). Such host regulation may be as great a determinant of tumor cell behavior *in vivo* as the specific oncogenic or tumor suppressor alterations occurring within the malignant cells themselves, and may be mediated by specific extracellular matrix molecules, growth factors, or host cells (Liotta *et al.*, 1991; Safarians *et al.*, 1996). Both positive (fibroblast, and endothelial cell) and negative (tumor infiltrating lymphocyte and macrophage) cellular regulators exist which profoundly affect tumor cell behavior *in vivo*

(Folkman and Klagsbrun, 1987; Cornil *et al.*, 1991). One host cell, the myoepithelial cell, has escaped attention. The myoepithelial cell, which lies on the epithelial side of the basement membrane, contributes largely to the synthesis and remodeling of this structure. This cell lies in juxtaposition to normally proliferating and differentiating epithelial cells in health and to abnormally proliferating and differentiating epithelial cells in precancerous disease states such as ductal carcinoma *in situ* (DCIS) of the breast. This anatomical relationship suggests that myoepithelial cells may exert important paracrine effects on normal glandular epithelium and may negatively regulate the progression of DCIS to invasive breast cancer. Previous studies of our laboratory have demonstrated that the human myoepithelial cell exerts multiple tumor suppressive effects on breast carcinoma cells inhibiting both cellular invasion and proliferation as well as inducing apoptosis (Sternlicht *et al.*, 1996a,b, 1999; Shao *et al.*, 1998). The inhibition of invasion is mediated predominantly by myoepithelial maspin (Sternlicht *et al.*, 1997; Shao *et al.*, 1998). In these previous studies (Sternlicht *et al.*, 1996a,b, 1997; Shao *et al.*, 1998), we used our established immortalized human myoepithelial cell lines and transplantable xenografts derived from benign or low grade human myoepitheliomas of the salivary gland, bronchus and breast. These cell lines and xenografts, though transformed, express nearly identical myoepithelial markers and gene products as their normal *in situ* counterparts and display an essentially normal diploid karyotype (Sternlicht *et al.*, 1996b). Furthermore they have maintained strong myoepithelial marker expression of S100 protein, smooth muscle actin, and cytokeratins over 100 passages. Unlike the vast majority of human tumor cell lines and xenografts which exhibit matrix-degrading properties these myoepithelial lines/xenografts like their normal myoepithelial counterparts *in situ* retain the ability to secrete and accumulate an abundant extracellular matrix composed of both basement membrane and non-basement membrane components. When grown as monolayers these myoepithelial cell lines exert profound and specific effects on normal epithelial and primary carcinoma morphogenesis (Sternlicht *et al.*, 1996b). These studies support that our established myoepithelial lines/xenografts recapitulate a normal differentiated myoepithelial phenotype and can therefore be used experimentally as a primary myoepithelial surrogate.

Because one other important cornerstone of tumor suppression would be a suppressive effect on angio-

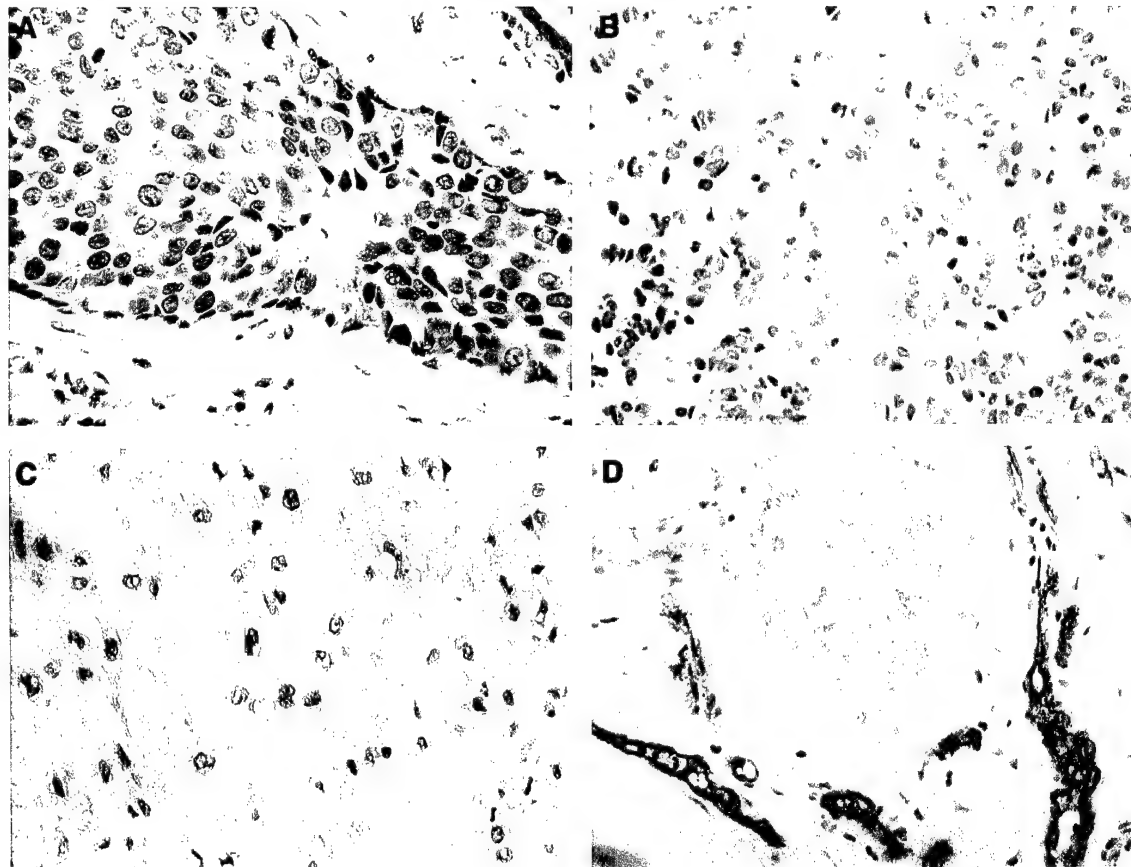
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genesis and because the main product of the myoepithelial cell, the basement membrane, is thought to be a natural reservoir of angiogenic inhibitors (Folkman, 1990, 1995b; Weidner *et al.*, 1991; Fridman *et al.*, 1991), we decided in the present study to investigate whether myoepithelial cells naturally display an anti-angiogenic phenotype. Because angiogenesis is now such an important biological phenomenon having implications in wound healing, inflammation and tumor biology, discovering and characterizing a new natural form of negative regulation of angiogen-

esis would have implications in all of these diverse areas of investigation.

Results

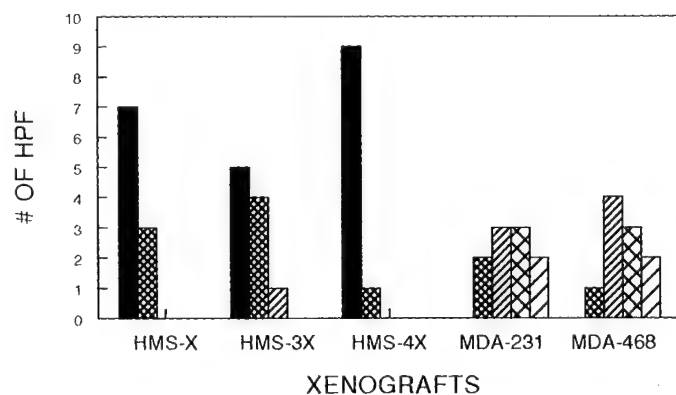
Myoepithelial cells *in situ* separate epithelial cells from stromal angiogenesis and this seemingly banal observation serves to illustrate the fact that stromal angiogenesis never penetrates this myoepithelial barrier (Figure 1a) raising the hypothesis that myoepithelial cells are



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vWf DENSITY

0 1-5 6-10 11-30 >30



natural suppressors of angiogenesis. This observation was re-inforced by a microscopic, immunohistochemical and DNA analysis of our myoepithelial xenografts. Our diverse myoepithelial xenografts secrete and accumulate an abundant extracellular matrix which is devoid of blood vessels in routine hematoxylin and eosin staining (Figure 1b) and vWf immunocytochemical staining (Figure 1c) in contrast to non-myoepithelial xenografts which show bursts of blood vessels (Figure 1d). Quantitation of vessel density in 10 H.P.F.'s reveals absent to low vessel density in the myoepithelial xenografts compared to the non-myoepithelial xenografts ($P < 0.01$) (Figure 1e). Murine DNA Cot-1 analysis further reveals the absence of a murine component in the myoepithelial xenografts. Since in the xenografts, angiogenesis would be murine in origin, the absence of a murine DNA component is another indication that angiogenesis is minimal. Interestingly the myoepithelial xenografts grew slowly

compared to the non-myoepithelial xenografts (Figure 1f), a feature which was not found in comparisons between the myoepithelial *versus* non-myoepithelial cell lines (data not shown).

To explain these *in vivo* observations, we analysed the gene expression profiles of our myoepithelial cell lines *versus* non-myoepithelial cell lines with respect to known angiogenic inhibitors and angiogenic factors. HMS-1, as a prototype myoepithelial cell line, constitutively expressed none or very low levels of the known angiogenic factors including bFGF, aFGF, angiogenin, TGF α , TGF β , TNF- α , VEGF, PD-ECGF, PlGF, IF α , HGF, and HB-EGF but rather expressed thrombospondin-1, TIMP-1 and soluble bFGF receptors at high levels; this was in contrast to a high angiogenic factor (which included bFGF, VEGF, TGF α , TGF β , HB-EGF, and PD-ECGF) to angiogenic inhibitor gene expression profile which was observed in non-myoepithelial cell lines (Figure 2a). Other

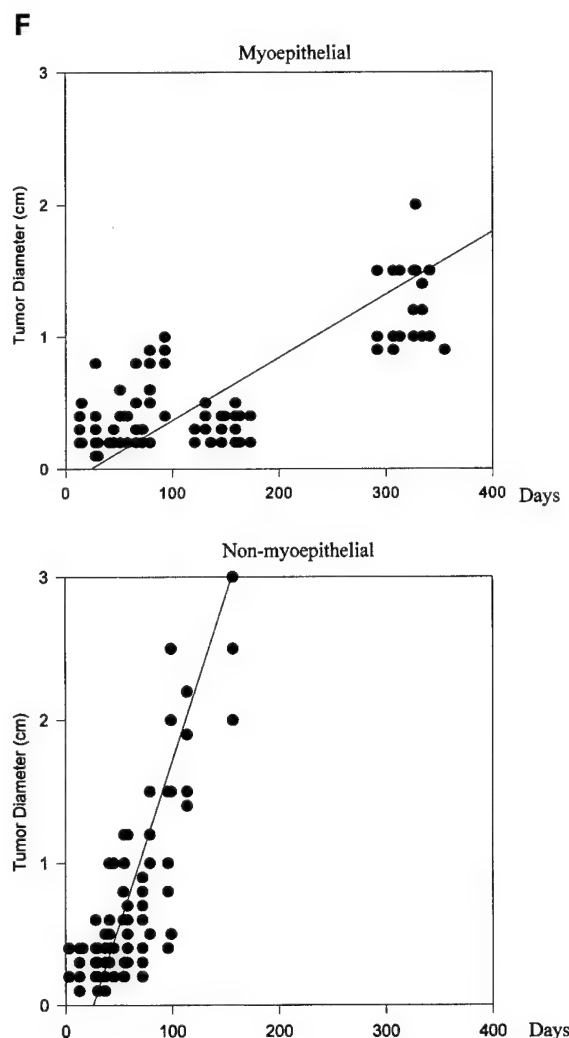


Figure 1 (a) A layer of myoepithelial cells immunoreactive for maspin demarcates the separation of proliferating epithelial cells from underlying stroma in this case of DCIS. Angiogenesis which occurs in the stroma never penetrates this layer of myoepithelial cells. Human myoepithelial xenografts, for example, HMS-X (b) exhibit an abundant extracellular matrix devoid of blood vessels. Absent vWf immunoreactivity is depicted in HMS-4X (c) compared to strong vWf immunoreactivity in the non-myoepithelial xenograft, MDA-MB-231 (d). Density of vWf positive vessels in 10 H.P.F.'s of myoepithelial *versus* non-myoepithelial xenografts reveals absent to significantly fewer blood vessels in the former xenografts (e). Growth rates of myoepithelial xenografts, for example, HMS-X, compared to growth rates of non-myoepithelial xenografts, for example, MDA-MB-231, revealed comparatively slow myoepithelial growth (f), suggesting a link to endogenously low levels of angiogenesis

myoepithelial cell lines (HMS-2-6) exhibited an angiogenic inhibitor/angiogenic factor profile similar to that of HMS-1. Interestingly in 2 M urea extracts of the myoepithelial xenografts but not in any of the non-myoepithelial xenografts, strong thrombospondin-1, TIMP-1 as well as plasminogen and prolactin fragments could be detected by Western blot (Figure 2a). HMS-1 and HMS-1 CM (concentrated 10–100-fold) exerted a marked inhibition of endothelial migration (Figure 2b) and proliferation (Figure 2c), both of which were abolished by pretreatment of the myoepithelial cells with cyclohexamide or dexamethasone. HMS-1 cells themselves did not migrate in response to either K-SFM, FCS, or bFGF. When mixed with

UVE, HMS-1 cells reduced endothelial migration to $12 \pm 6\%$ of control ($P < 0.01$). HMS-1 concentrated CM reduced migration to $8 \pm 7\%$ of control ($P < 0.01$). All of the non-myoepithelial malignant human cell lines studied stimulated both endothelial migration and proliferation. Concentrated CM from HMS-1, when fractionated on a heparin-Sepharose column, inhibited endothelial proliferation to $47 \pm 10\%$ of control ($P < 0.01$). This inhibitory activity was present only in the 1.5–2.0 M gradient fraction (Figure 2d). Pretreatment of HMS-1 cells with PMA resulted in a 2–5-fold increase in endothelial antiproliferative inhibitory activity in both unfractionated CM (Figure 2c) as well as in the heparin-Sepharose fraction. Western blot of

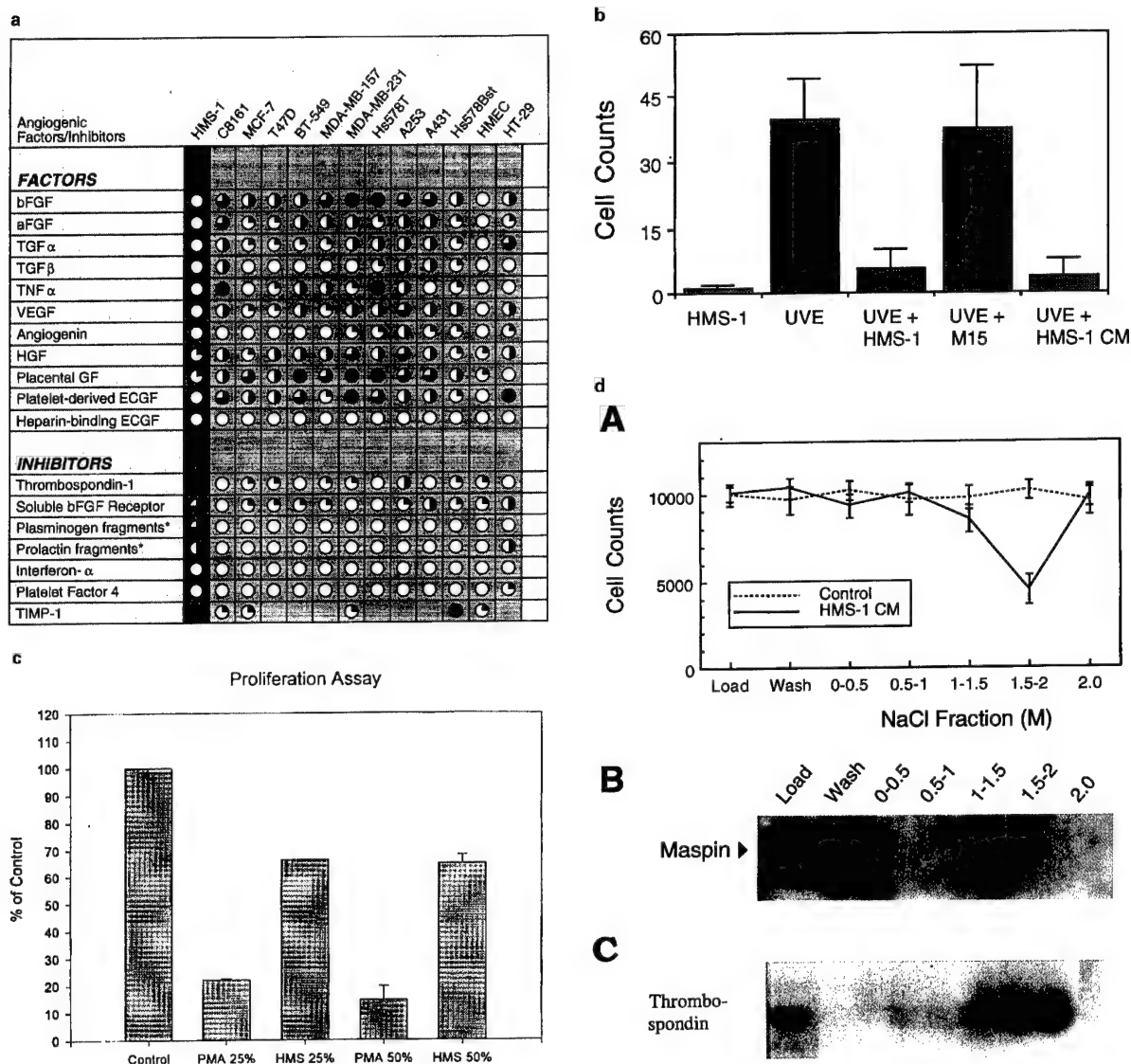


Figure 2 (a) Relative constitutive gene expression profiles of diverse angiogenic inhibitors and angiogenic factors in HMS-1 compared to numerous other non-myoepithelial cell lines. All measurements were made by Western blot on either CM or matrix extracts* and depicted as relative levels of expression. HMS-1 (HMS-X*) uniquely expressed a balance of angiogenic inhibitors over angiogenic factors. (b) Inhibition of UVE chemotaxis by HMS-1 cells and HMS-1 CM is depicted as cell counts collected on the undersurface of a dividing filter. HMS-1 cells themselves were non-migratory. A control non-myoepithelial human melanoma cell line, M15, did not inhibit UVE chemotaxis. (c) HMS-1 CM (percentage of CM added) inhibition of UVE proliferation is depicted. PMA pretreatment of HMS-1 increased antiproliferative activity. (d) Fractionation of the HMS-1 CM on a heparin-Sepharose column revealed peak antiproliferative activity in the 1.5–2.0 NaCl fraction [A], a fraction devoid of maspin [B] but containing thrombospondin-1 [C]

the heparin-Sepharose column fractions revealed the presence of maspin in the load and wash fractions only and not in the 1.5–2.0 M fraction; in contrast the 1.5–2.0 M NaCl fraction contained thrombospondin-1 (Figure 2d). Immunoprecipitation of this fraction with anti-thrombospondin was effective at removing all thrombospondin-1 but decreased endothelial antiproliferative activity by only 50% raising the possibility that other angiogenic inhibitors as yet uncharacterized were present in this fraction. The other myoepithelial cell lines (HMS-2-6) exhibited similar anti-angiogenic inhibitory activity in their fractionated and unfractionated CM.

To further explain our *in vivo* observations of minimal angiogenesis in our myoepithelial xenografts, *in vitro* and *in vivo* hypoxia studies were carried out. Non-myoepithelial xenografts, e.g. MDA-MB-231 exhibited florid hypoxia but only minimal necrosis (Figure 3a) when they reached a size of 2.0 cm. In contrast, the myoepithelial xenografts exhibit only minimal hypoxia but prominent necrosis ($P < 0.001$) (Figure 3b,c) at the same size of 2.0 cm. Quantitation of the areas of hypoxia (pimonidazole immunoreactivity) and areas of necrosis (Figure 3d) in the myoepithelial *versus* non-myoepithelial xenografts suggest that in the myoepithelial tumors where angiogenesis is minimal hypoxic areas progress to necrosis rapidly whereas in the non-myoepithelial tumors hypoxic areas accumulate but do not progress to necrosis presumably from the angiogenesis which the hypoxia elicits. Comparative analysis of myoepithelial *versus* non-myoepithelial cell lines to low O_2 tension reveals that while both cell lines sense hypoxia in that they respond by increasing HIF-1 α (Figure 3e), the myoepithelial lines upregulate their steady state mRNA levels of the downstream genes, VEGF (Figure 3f) and iNOS (Figure 3g) to a lesser extent than the carcinoma lines suggesting the possibility of decreased transactivation of HRE. Specifically we observed an approximate 1.7-fold increase in VEGF (1.1-fold increase in iNOS) in myoepithelial cells in response to hypoxia compared to an approximate 2.5-fold increase in VEGF (1.5-fold increase in iNOS) in carcinoma cell lines in response to hypoxia. Although these fold differences by themselves are not impressive, the absolute levels of VEGF (and iNOS) expressed in carcinoma cells in response to hypoxia are 2.5-fold greater for VEGF (and 1.7-fold greater for iNOS) than the levels of VEGF (and iNOS) expressed in myoepithelial cells in response to hypoxia. Therefore it can be concluded that myoepithelial cells do not express VEGF or iNOS in response to hypoxia to nearly the same extent as carcinoma cells.

To study both local and systemic effects of myoepithelial cells on metastasis, spontaneously metastasizing tumor cells were injected into our myoepithelial xenografts. The highly metastatic *neoC8161* cells injected into the myoepithelial xenografts could be recovered in significant numbers although the numbers of clones recovered were less than those recovered from the nonmyoepithelial xenografts. Histological analysis of the extirpated xenografts revealed *neoC8161* cells actively invading through all of the nonmyoepithelial xenografts in contrast to the appearance within the myoepithelial xenografts where the *neoC8161* cells were confined to the immediate areas around the injection site. Pulmonary metastases of *neoC8161* were comple-

tely absent in the myoepithelial xenograft-injected group whereas they were quite numerous in the nonmyoepithelial group ($P < 0.001$). Analysis of extirpated myoepithelial xenografts containing injected *neoC8161* cells contained no evidence of murine angiogenesis by either vWf immunocytochemical studies or murine DNA Cot-1 analysis whereas a similar analysis of extirpated *neoC8161* injected-non-myoepithelial xenografts showed an increase in murine angiogenesis by both methods (data not shown). This suggested that either the matrices of our myoepithelial xenografts or gene product(s) of the myoepithelial cells or both were inhibiting *neoC8161*-induced angiogenesis *in vivo*. We, in fact, found evidence of thrombospondin-1, TIMP-1, soluble bFGF receptors, prolactin and plasminogen fragments within 2 M urea extracts of our myoepithelial xenografts (Figure 4a). In tail vein injection studies of *neoC8161*, in mice harboring the myoepithelial xenografts, *neoC8161* formed smaller pulmonary colonies than in mice harboring non-myoepithelial xenografts or in control mice (no xenografts) ($P < 0.01$) (Figure 4b,c,d). In a vWf factor immunocytochemical analysis of these smaller colonies in the mice harboring the myoepithelial xenografts, angiogenesis was minimal (data not shown).

Discussion

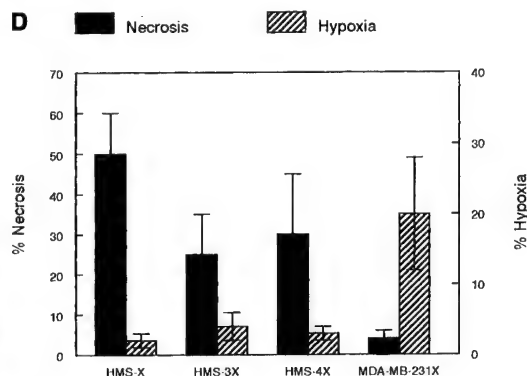
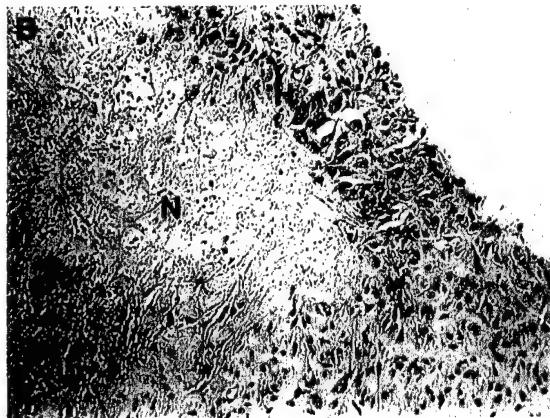
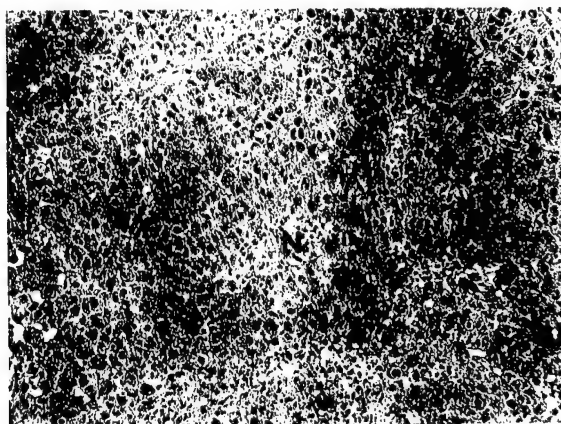
Blood vessel proliferation (angiogenesis) occurs in a number of disease states including inflammation and neoplasia and both positive and negative regulators of angiogenesis exist that influence this process. However angiogenesis is not ubiquitous and certain tissues such as mature cartilage are resistant to this process. Angiogenesis also does not occur on the epithelial side of the basement membrane even in proliferative and precancerous disease states which stimulate angiogenesis in the underlying stroma. The border between epithelial cells and stroma is lined by myoepithelial cells suggesting that these cells may exert negative regulation on the angiogenic process. Our studies, in fact, provide evidence that human myoepithelial cells are natural suppressors of angiogenesis and display this phenotype in a multifaceted manner.

Tumor angiogenesis, like tumor invasion, is thought to be determined by the balance of natural positive and negative regulators which occur within the tumor's microenvironment (Tobacman, 1997; Xiao *et al.*, 1999; Folkman, 1995a; Hanahan and Folkman, 1996). Although certain specific angiogenic inhibitors can be expressed by malignant cell lines, the vast majority of malignant cell lines secrete both more angiogenic factors than inhibitors and a greater molar ratio of angiogenic factors to inhibitors. Situations, both natural and therapeutic, which shift the overall balance to an excess of angiogenic inhibitors over angiogenic factors would be situations which are tumor suppressive. All of our myoepithelial cell lines constitutively expressed a high ratio of angiogenic inhibitors to angiogenic factors. The net effects of myoepithelial cells and their CM inhibited endothelial migration; in contrast the net effects of the non-myoepithelial malignant human cell lines studied all stimulated endothelial migration and proliferation. Heparin-Sepharose fractionated CM from our myoepithelial cell

lines demonstrated a marked inhibition of endothelial proliferation in the 1.5–2.0 M NaCl gradient fraction, a fraction containing thrombospondin-1 instead of maspin. Thrombospondin immunoprecipitation experiments performed on this fraction suggested that the mechanism of antiangiogenesis, in part, involved thrombospondin-1. It should be pointed out that our assays for antiangiogenic activity utilized human umbilical vein endothelial cells (commonly called HUVECs), cells which lack one of the receptors, CD36, to which thrombospondin-1 binds (Dawson *et al.*, 1997). However another study found proliferation in HUVECs to be inhibited by thrombospondin-1 (Bagavandoss and Wilkes, 1990). The implication of these studies to our present work is that our use of HUVECs might have resulted in an underestimate of the thrombospondin-1 mediated antiangiogenic activity of CM from myoepithelial cell lines. Our use of microvascular endothelial cells might be more appropriate for our future studies. It is also interesting that in our human umbilical vein endothelial proliferation assays, maspin did not appear to have antiangiogenic activity. Maspin has recently been observed, in fact, to be an angiogenesis inhibitor (Zhang *et al.*, 2000). In that study, the antiangiogenic effects of maspin were observed against human microvascular endothelial cells and not human umbilical vein endothelial cells. Our previous studies have shown that myoepithelial cells secrete high levels of maspin both *in vitro* and *in vivo* (Sternlicht *et al.*, 1997). Myoepithelial maspin can hence be added to the growing list of angiogenic inhibitors produced selectively and at high levels by

myoepithelial cells. Interestingly both maspin and thrombospondin-1 have been shown to be autocrine tumor suppressors in experiments involving their respective cDNAs transfected into breast carcinoma cell lines (Zou *et al.*, 1994; Weinstat-Saslow *et al.*, 1994). In our past and present studies we argue for a role of both molecules as paracrine tumor suppressors elaborated by myoepithelial cells. The similarly enhancing effect of PMA on both invasion inhibition demonstrated in a previous study (Sternlicht *et al.*, 1997) and angiogenesis inhibition demonstrated in the present study argues that PMA pleiotropically promotes the natural suppressor effects of myoepithelial cells.

There was minimal angiogenesis in our myoepithelial xenografts which also exhibited a very slow growth rate compared to their non-myoepithelial counterparts. This growth rate difference was not observed between the myoepithelial *versus* non-myoepithelial cell lines. It is attractive then to postulate that the slow growth of the myoepithelial xenografts is causally related to their minimal angiogenesis. To explain this minimal myoepithelial angiogenesis, we reasoned that there were three possible mechanisms. The first was that myoepithelial cells expressed a balance of angiogenic inhibitors over angiogenic factors so that the myoepithelial tumor's microenvironment was inhibitory to angiogenesis. The second possible mechanism was that myoepithelial cells, like chondrocytes, either do not sense hypoxia or are resistant to the effects of hypoxia. The third possible mechanism was that myoepithelial cells



experience hypoxia but are not able to respond to it by inducing angiogenesis. Our studies revealed that both the first and third mechanisms are operating within myoepithelial cells but that the second

mechanism is not: myoepithelial cells secrete a balance of angiogenic inhibitors over angiogenic factors, they do however sense hypoxia but are not able to respond effectively downstream to hypoxia.

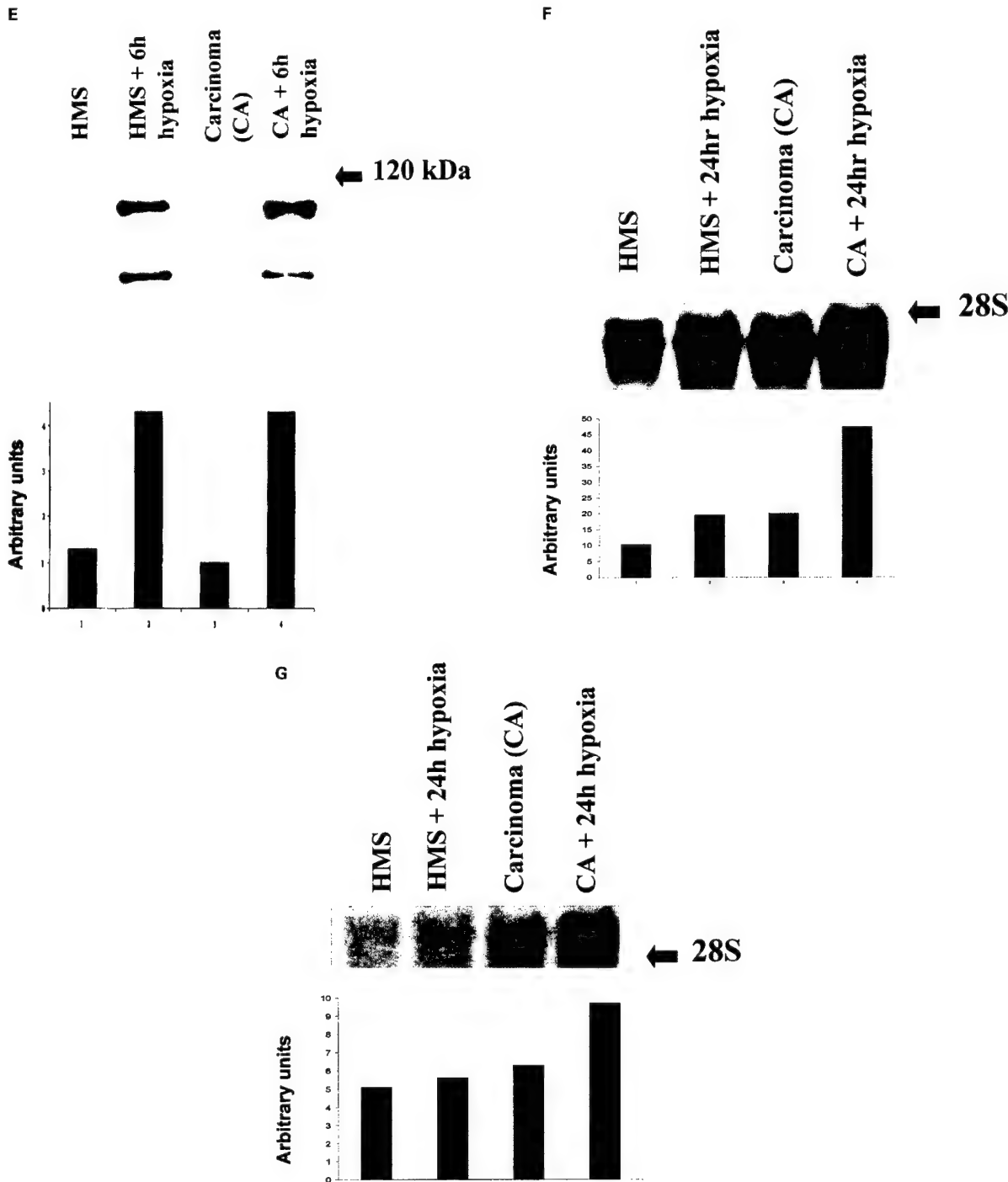


Figure 3 Pimonidazole immunoreactivity as a measure of hypoxia is prominently contrasted in a non-myoepithelial xenograft, the MDA-MB-231 (a), versus two myoepithelial xenografts, HMS-4X (b) and HMS-X (c). Only a thin rim of pimonidazole immunoreactivity (H) is present in the myoepithelial xenografts but instead large adjacent areas of frank necrosis (N) are conspicuous (b)(c). Necrosis (N) in the MDA-MB-231, in contrast, is inconspicuous but hypoxia (H) is prominent (a). Per cent hypoxia and per cent necrosis in the myoepithelial versus non-myoepithelial xenografts are contrasted (d). In the myoepithelial xenografts necrosis is prominent whereas hypoxia is inconspicuous where the reverse is true in the non-myoepithelial xenografts. Under low O_2 tension, myoepithelial cells, e.g. HMS-1 (HMS), like non-myoepithelial carcinoma cells, e.g., MDA-MB-231 (CA), show an increase in HIF-1 α (e) but, unlike carcinoma cells (CA), show less of an increase in VEGF (f) and iNOS (g) steady state mRNA levels. Other myoepithelial and carcinoma lines tested exhibited a similar pattern of findings

The specific gene products of myoepithelial cells per se may not be the sole determinants of this cell's antiangiogenic phenotype because these gene products

may undergo extracellular modifications. We have previously demonstrated, for example, that the proteinase inhibitor, PNII undergoes extracellular *in vivo*

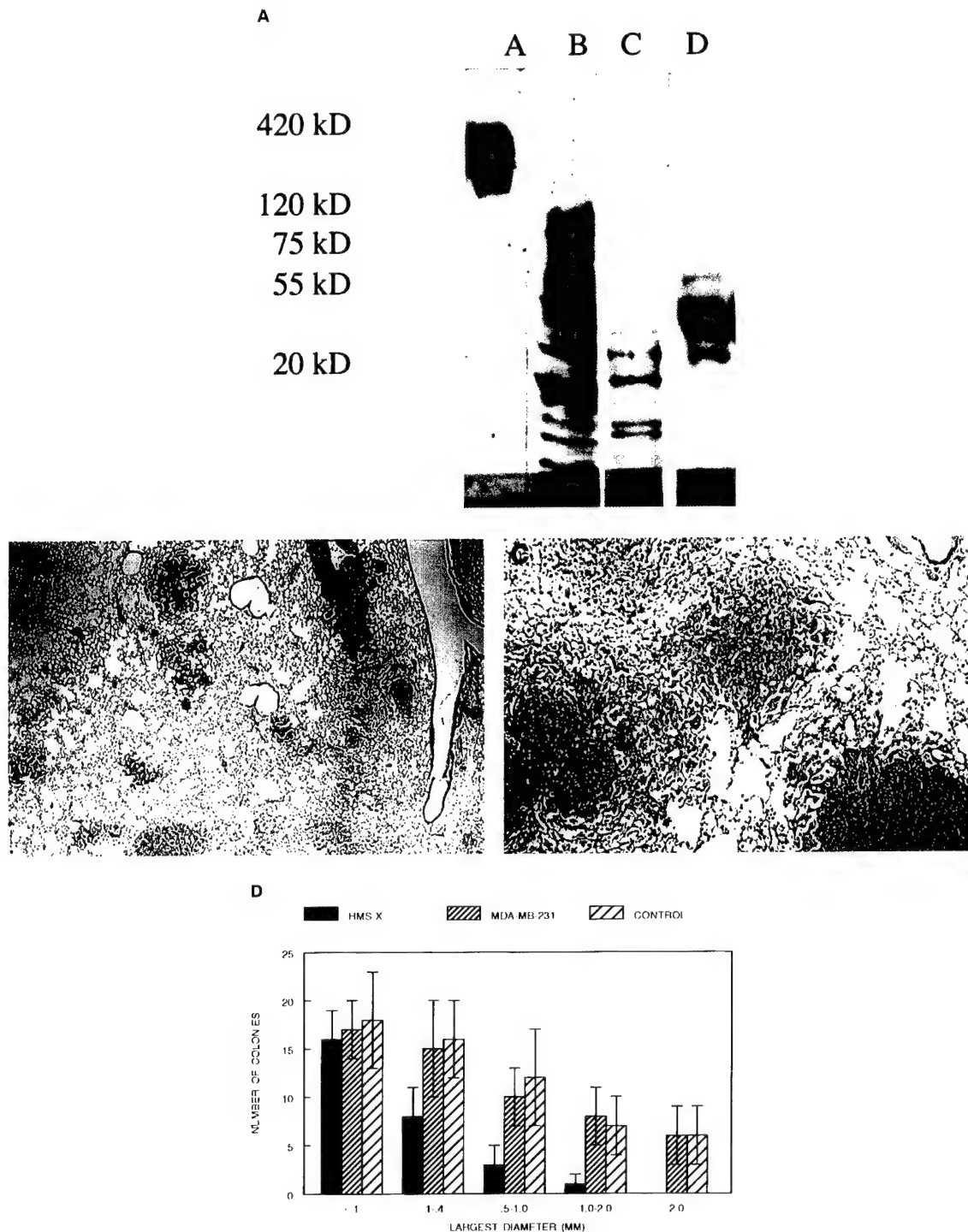


Figure 4 (a) Extraction of myoepithelial cell matrix reveals a number of sequestered angiogenic inhibitors and fragments: [A] thrombospondin-1, [B] soluble bFGF receptors, [C] prolactin fragments and [D] plasminogen fragments. (b,c,d) Differences in hematogenous pulmonary metastases with tail vein injected *neoC8161* is in evidence in mice harboring myoepithelial xenografts (b) versus non-myoepithelial xenografts (c) versus control (no xenografts). The number and size of metastatic colonies in mid-longitudinal cross section of lung was determined by digital image analysis and expressed as mean \pm standard error. Quantitation of pulmonary metastases revealed similar numbers of colonies in all three groups but a marked decrease in size in the group harboring the myoepithelial xenografts (d). Results depict a representative myoepithelial xenograft, HMS-X, a representative non-myoepithelial xenograft, MDA-MB-231, and control (no xenograft). Other myoepithelial and non-myoepithelial xenografts recapitulated these results

processing within the matrices of HMS-X and HMS-4X to a novel 95 kDa fragment retaining full proteinase inhibitor activity and found bound to the myoepithelial matrix (Sternlicht *et al.*, 1996a, 1997). Interestingly when we screened myoepithelial cell CM by Western blots for angiogenic inhibitors, we did not observe evidence of plasminogen or prolactin secretion in the majority of our myoepithelial lines. However when we extracted our myoepithelial xenografts with 2 M urea we found evidence of processed fragments of plasminogen and prolactin in all of our myoepithelial xenografts. Whether these fragments represent angiogenic inhibitors (the 38 kDa angiostatin or the 16 kDa prolactin fragment) (O'Reilly *et al.*, 1994; Clapp *et al.*, 1993) remains to be determined. Since most of our myoepithelial cell lines do not secrete the parental molecules *in vitro*, the fragments are likely derived from circulating murine parental molecules which are sequestered by the anionic myoepithelial matrix and processed. There are therefore two types of molecules present within the myoepithelial matrix, molecules secreted by the myoepithelial cells themselves, e.g. thrombospondin-1 and TIMP-1 and molecules sequestered from the circulation, e.g. prolactin and plasminogen and processed. These latter molecules perhaps serve as substrates for unknown enzymes produced by myoepithelial cells. Certain other cell types such as small cell carcinoma can sequester within their stroma, for example, circulating antithrombin and subsequently cleave this molecule to produce a conformationally altered molecule which has antiangiogenic activity (O'Reilly *et al.*, 1999). Myoepithelial cells with their abundant and anionically charged stroma may similarly sequester and process circulating molecules into highly antiangiogenic forms.

Highly metastatic *neo*C8161 cells when injected into the matrices of our myoepithelial xenografts predictably then were not able to stimulate angiogenesis nor metastasize. Since viable *neo*C8161 cells could still be recovered from the xenografts, it likely is the bound angiogenic inhibitors, e.g. thrombospondin-1 and TIMP-1 and possibly the processed fragments of the parental plasminogen and prolactin originally derived from the circulation that are responsible for the local anti-angiogenic effects. Our myoepithelial xenografts were also able to suppress distant pulmonary metastases after hematogenous dissemination of tumor cells following intravenous injection. It is attractive to postulate that this systemic effect on metastasis inhibition is mediated through the release rather than the sequestration of angiogenic inhibitors. One implication then of our *in vivo* findings is that myoepithelial xenografts give rise to circulating inhibitors of angiogenesis. Another possibility to explain our observations is that our myoepithelial xenografts sequester and remove circulating angiogenic factors from serum. Both phenomenon would be anti-angiogenic. We are presently screening serum and urine for antiangiogenic activity and have observed in preliminary studies inhibition of endothelial proliferation *in vitro* with both serum and concentrated urine from mice harboring myoepithelial xenografts. We will be attempting to purify this angiogenic inhibitory activity in the near future.

Past studies have shown that different human and murine cell lines and xenografts produce different

angiogenic inhibitors (O'Reilly *et al.*, 1994, 1997, 1999). Myoepithelial cell lines and xenografts exhibit unique anti-angiogenic properties, suggesting that they may represent a natural source of angiogenic inhibitors yet undiscovered.

Materials and methods

Cell lines and xenografts

Informed patient consent and certification from the UCLA Human Subject Protection Committee was obtained. Approval from the Chancellor's Animal Research Committee was also requested and obtained (certification ARC 95-127-11). Early passage (passage 10–15) human myoepithelial cell lines/xenografts: HMS-1-6, X-6X established in our laboratory (Sternlicht *et al.*, 1996a,b, 1997; Shao *et al.*, 1998) were used. Other non-myoepithelial cell lines/xenografts used included the human melanomas, C8161 (Dr Mary Hendrix, University of Iowa) and M15 (Dr Don Morton, John Wayne Cancer Center, Santa Monica, CA, USA); the human breast carcinomas, MDA-MB-231, MDA-MB-468, MCF-7, T47D, BT-549, MDA-MB-157, Hs578T, Hs578Bst; other malignant lines including the A431, vulvar carcinoma, A253, salivary gland carcinoma and HT-29, colon carcinoma (American Type Culture Collection, Rockville, MD, USA). The highly metastatic C8161 line was transfected by us with pSV2neo in a previous study (Safarians *et al.*, 1996). Normal cells used included human mammary epithelial cells (HMEC) and human umbilical vein endothelial cells (UVE) (Clonetics, San Diego, CA, USA). Serum free conditioned media (CM) was collected from many of these lines over 24 h and concentrated 10–100-fold using Centrprep-10 concentrators (Amicon, Beverly, MA, USA). Prior to collection of CM, myoepithelial cells were also pretreated with: cyclohexamide (CHX) (40 µg/ml) for 24 h; phorbol 12-myristate 13-acetate (PMA) (5 µM) for 8 h; dexamethasone (0.25 µM) for 24 h. The growth of the different myoepithelial xenografts was observed in 4 week old female athymic/scid mice and compared to the growth of non-myoepithelial xenografts.

Antibodies and probes

High molecular weight genomic DNA was extracted from the myoepithelial and non-myoepithelial xenografts and dot blotted and probed with a murine specific α -³²P-dCTP-labeled murine Cot-1 DNA probe (Life Technologies, Inc., Gaithersburg, MD, USA) to determine the murine DNA component of the xenografts (Alpaugh *et al.*, 1999).

Angiogenic factors, angiogenic inhibitors and other relevant molecules were profiled by Western blot analysis of concentrated CM of the cell lines and 2 M urea extracts of the xenografts according to established methods (Sternlicht *et al.*, 1996a, 1997). Western blots were performed as previously described using the manufacturers' recommended dilutions for primary antibodies and a 1:50 000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL, USA) followed by development of the reaction with the ECL detection system (Amersham Life Sciences, Arlington Heights, IL, USA). Primary rabbit polyclonal antibodies to maspin were obtained (PharMingen, San Diego, CA, USA). Primary antibodies to known angiogenic factors and angiogenic inhibitors included mouse monoclonals or rabbit polyclonals to bFGF, aFGF, angiogenin, TGF α , TGF β , TNF- α , VEGF, PD-ECGF, PlGF, IF α , HGF, HB-ECGF and IF- α (all from R&D Systems, Minneapolis, MN, USA); plasminogen and PF4 (American Diagnostica, Inc., Greenwich, CT, USA); thrombospondin-1 (Sigma Bio Sciences, St. Louis, MO, USA); rabbit anti-rTIMP-1, (Dr Judith C

Gasson, UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA, USA); prolactin (Dr Richard Weiner, University of California, San Francisco, CA, USA); and bFGF soluble receptor (Dr Anne Hanneken, Scripps Research Institute, LaJolla, CA, USA). Relative levels of expression of the angiogenic factors and angiogenic inhibitors were expressed as 'pie slices'. The pie slices refer to the semiquantitative (relative) levels of expression of a given factor in different (myoepithelial *versus* non-myoepithelial) cell lines. Because of differing affinities of primary antibodies, the size of the pie slices can not be compared among factors. Scion Image software was used for densitometric analyses of the bands and quantification of the signal on Western blots developed by ECL. Also used was a primary rabbit antibody to identify endothelial cells (blood vessels): anti-von Willibrand factor (vWf) (DAKO, Carpinteria, CA, USA). Standardized immunoprecipitation protocols using Sepharose-protein A (Langone, 1982) were employed in the evaluation of thrombospondin-1 activities.

Formalin-fixed paraffin-embedded tissues of human myoepithelial and non-myoepithelial xenografts at a size (1.0 cm diameter) where necrosis was minimal were incubated with rabbit antibodies to vWf (1:1000). Peroxidase-conjugated goat anti-rabbit IgG was used as secondary antibody at 1/200 dilution. Colorimetric detection of peroxidase-conjugated secondary antibody was with diaminobenzidine. The number of vWf profiles per high power field (H.P.F.) in 10 high power fields (H.P.F.'s) from each xenograft was determined.

In vitro angiogenic chemotaxis and proliferation assays

Standard endothelial migration assays were carried out over 4 h using a chemotaxis chamber (Neuroprobe, Cabin John, MD, USA) with 10 ng/ml bFGF as chemoattractant (Nguyen *et al.*, 1993). Effects of HMS-1-6 CM (concentrated 10–100-fold) and HMS-1-6 cells added to the upper compartment were tested. Standard endothelial proliferation assays (O'Reilly *et al.*, 1997) were also carried out over 72 h with 25 µg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA). Heparin-Sepharose NaCl gradient fractionated HMS-1-6 CM was analysed for inhibitory activity.

In vitro hypoxia studies

To establish an hypoxic environment (Namiki *et al.*, 1995), cells were placed in a Modular Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA), and a pre-analysed air mixture (1% O₂, 5% CO₂, and 94% N₂) was flushed through the air-tight chamber for 15 min twice a day. The chamber was then placed in a 37°C incubator, and hypoxia was maintained for 6–24 h. After an initial 6 h period of hypoxia, the cells were harvested and their nuclear fraction collected as follows: Cells were harvested and thawed on ice and reconstituted in 5 volumes of buffer A (10 mM Tris, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl) with freshly added supplements (1 M DTT, 0.2 M PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 0.5 M Na₃VO₄). The suspension was then passed through a pre-cooled homogenizer using 25 strokes to lyse the cells. The lysate was then centrifuged for 10 min at 4°C, and the supernatant collected and stored as the cytoplasmic fraction. The pellet was then resuspended in 3.5 volumes of buffer C (0.42 M KCl, 20 mM Tris, pH 7.5, 1.5 mM MgCl₂, 20% glycerol) with the same supplements as above freshly added. The suspension was then centrifuged for 30 min at 4°C, and the supernatant was collected as the nuclear fraction. Western blot analysis on this nuclear fraction for HIF-1α was performed using a monoclonal antibody to HIF-1α (Lab Vision Corporation, Fremont, CA, USA). After a 24 h period of hypoxia, the cells were harvested, total RNA extracted and 20 µg separated on denaturing agarose gels, transferred to nylon membranes, and

probed with a-³²P-dCTP-labeled probes using standard Northern blot protocols. cDNA probes used included VEGF (Dr Howard Reber, UCLA) and iNOS (Dr Gautam Chaudhuri, UCLA). The 36P4 probe (Dr Judith Berliner, UCLA) was used as a housekeeping probe.

In vivo hypoxia studies

The principle that pimonidazole binds to thiol-containing proteins specifically in hypoxic cells (Varia *et al.*, 1998) was exploited by immunocytochemical detection of pimonidazole using a mouse monoclonal antibody (Dr James Raleigh, UNC, Chapel Hill, NC, USA) in tissue sections of the myoepithelial *versus* the non-myoepithelial xenografts grown in mice receiving intraperitoneal injections of pimonidazole hydrochloride (0.5 g/m²) followed by extirpation of the xenografts 24 h later and immunocytochemical analysis. Pimonidazole immunoreactivity was present as cytoplasmic staining. The number of hypoxic cells (positively stained) was expressed as a percentage of total. Areas showing frank necrosis were also observed and quantitated. Care was taken in these comparative studies to use xenografts of equal size; for these studies xenografts measuring 2.0 cm in diameter were used.

In vivo angiogenesis and metastasis studies

The myoepithelial xenografts (HMS-X, HMS-3X and HMS-4X) and non-myoepithelial xenografts (MDA-MB-231, MDA-MB-468) were established in nude and scid mice and allowed to grow to a size of 1.0 cm. This size was chosen because there was no appreciable necrosis in any of the xenografts at this size. 10⁵ neoC8161 cells suspended in 50 µl Hank's Balanced Salt Solution (HBSS) were injected into the centers of the xenografts and allowed to grow for 4–8 weeks. Random xenografts from each group were injected with 50 µl trypan blue instead of cells and immediately extirpated to verify that the injections had been delivered to the centers of the xenografts. After 4–8 weeks, animals were sacrificed and primary tumors and lungs were subjected to detailed histopathological examination, collagenase digestion, and culturing of the liberated cells in G418 sulfate (0.2 mg/ml) (Life Technologies, Inc., Gaithersburg, MD, USA). The number of recovered neoC8161 clones from each site was determined by phase-contrast image analysis of the cultured dishes. The extirpated xenografts were also studied immunocytochemically for evidence of vWf immunoreactivity and subjected to DNA extraction and murine DNA Cot-1 analysis to estimate the degree of murine angiogenesis. 10⁵ neoC8161 cells were also suspended in 100 µl HBSS and injected into the tail vein (hematogenous metastasis) in mice harboring 2 cm myoepithelial, non-myoepithelial or control (no xenografts). Animals were sacrificed 4 weeks following injection. Removed lungs were inflated, paraffin embedded, sectioned and stained. Both colony number and size (greatest diameter dimension)/mid-longitudinal cross-section of lung were tabulated by digital image analysis, which utilized a Leitz Dialux microscope linked to a Vidicon camera, an IBM PC with PCVision digitizer, and Microscience software. All experiments were performed with groups of 10 mice. Results were analysed with standard tests of statistical significance, including the 2-tailed Student's *t*-test and a one-way analysis of variance (ANOVA).

Abbreviations

UVE, human umbilical vein endothelial cells; CM, conditioned medium; FCS, fetal calf serum; DCIS, ductal carcinoma *in situ*; TIMP-1, tissue inhibitor of metalloproteinase-1; HMEC, human mammary epithelial cells; K-SFM, keratinocyte serum-free medium; vWf, von Will-

ebbrand factor; PMA, phorbol 12-myristate 13-acetate; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; TGF α , transforming growth factor α ; TGF β , transforming growth factor β ; TNF α , tumor necrosis factor α ; VEGF, vascular endothelial growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; PlGF, placental growth factor; IF α , interferon α ; HGF, hepatocyte growth factor; HB-EGF, heparin-binding endothelial growth factor; PF4, platelet factor 4; and iNOS, inducible nitric oxide synthase; HIF-

1 α , hypoxia inducible factor-1 α ; HRE, hypoxia response element.

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Myoepithelial-Specific CD44 Shedding Contributes to the Anti-invasive and Antiangiogenic Phenotype of Myoepithelial Cells¹

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Myoepithelial cells surround incipient ductal carcinomas of the breast and exert anti-invasive and antiangiogenic effects *in vitro* through the elaboration of suppressor molecules. This study examines one putative molecule, solubilized CD44 produced by myoepithelial shedding of membrane CD44. Studies with different human myoepithelial cell lines demonstrate that myoepithelial cells express and shed both the 85-kDa standard (CD44s) and the 130-kDa epithelial (CD44v8-10) isoforms, findings further confirmed by the use of isoform-specific antibodies. PMA pretreatment enhances CD44 shedding detected by two different methods at different time points: a reduction in surface CD44 at 2 h by flow cytometry and a marked decrease in both total cellular CD44 and plasma membrane CD44 at 12 h by Western blot. This shedding is both specific for CD44 and specific for myoepithelial cells. This shedding is inhibited by the chymotrypsin inhibitors chymostatin and α_1 -antichymotrypsin but not by general metallo-, cysteine, or other serine proteinase inhibitors. Myoepithelial-cell-conditioned medium and affinity-purified solubilized CD44 from this conditioned medium block hyaluronan adhesion and migration of both human carcinoma cell lines and human umbilical vein endothelial cells. © 2000 Academic Press

Key Words: myoepithelial cells; tumor suppression; CD44; anti-angiogenesis; anti-invasion.

INTRODUCTION

Myoepithelial cells which surround ductal epithelium of glandular organs such as the breast exert multiple paracrine suppressive effects on incipient cancers which arise from this epithelium [1–3]. This paracrine suppression may keep the genetic alterations occurring within malignant epithelial cells in check so that the

evolving cancer exists for a number of years only as an *in situ* lesion confined within the ductal system [4]. This *in situ* lesion is commonly termed ductal carcinoma *in situ* or DCIS. Because of their close proximity, myoepithelial cells would be anticipated to exert important paracrine influences on normal, precancerous, and cancerous epithelial cells. Myoepithelial cells of the breast differ from luminal ductal and acinar epithelial cells in many ways: they lack expression of the common hormonal receptor, ER- α , and its responsive genes like PR; they lie next to the basement membrane and contribute to the synthesis of that structure; they rarely transform or proliferate; and when they do give rise to only low-grade benign neoplasms [5, 6]. Myoepithelial cells in a sense can be regarded then as both autocrine and paracrine tumor suppressors. Our laboratory has established immortalized myoepithelial cell lines and transplantable xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3), breast (HMS-4, HMS-5), and bronchus (HMS-6) (with their respective xenografts designated as HMS-X) [1–3, 5, 6]. These cell lines and xenografts express identical myoepithelial markers as normal myoepithelial cells *in situ* and display an essentially normal diploid karyotype. In previous studies we have demonstrated that our myoepithelial cell lines/xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α_1 antitrypsin, an unidentified 31- to 33-kDa trypsin inhibitor, thrombospondin-1, soluble bFGF receptors, and maspin [1–3]. Our human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion and endothelial cell migration and proliferation (angiogenesis) *in vitro* [3, 7]. Our myoepithelial cell lines also inhibit breast carcinoma proliferation *in vitro* through an induction of breast carcinoma cell G₂/M arrest and apoptosis [3], the latter phenomenon of which also occurs *in situ* within DCIS [8].

In our studies demonstrating that myoepithelial cells inhibited both invasion and angiogenesis we noted that treatment with PMA potentiated both types of suppression [1, 7]. In examining possible mecha-

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nisms for these effects we surveyed our myoepithelial cell lines for PMA-induced changes in expression levels of general categories of molecules that had been implicated in tumor invasion and angiogenesis, namely proteinases/proteinase inhibitors, angiogenic factors/angiogenic inhibitors, and adhesion molecules. In the latter category we noted that PMA profoundly decreased the levels of surface CD44 on myoepithelial cells and we decided to study this phenomenon and its significance further in the present study.

MATERIALS AND METHODS

Cell lines. Human myoepithelial cell lines (HMS-1-6) from benign myoepithelial tumors of diverse origins had been previously established in our laboratory [1-3, 5, 6] and maintained in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50 μ g/ml) and recombinant human epidermal growth factor (5 ng/ml) (GIBCO-BRL, Gaithersburg, MD). Other nonmyoepithelial cell lines used included normal human mammary epithelial cells (HMECs) and normal human umbilical vein endothelial cells (HUVECs) (both from Clonetics, San Diego, CA), the human melanoma C8161 line (a gift of Dr. M. J. C. Hendrix, University of Iowa, Iowa City, IA), and estrogen-receptor-negative breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 (American Type Culture Collection, Rockville, MD). These latter cell lines were maintained in Eagle's MEM supplemented with 10% FBS (GIBCO-BRL) and penicillin-streptomycin antibiotics. Conditioned medium from the myoepithelial cell lines untreated and treated with PMA (5 μ M) (Sigma Chemical Co., St. Louis, MO) was collected in basal K-SFM and concentrated up to 10-fold with Centriprep-10 concentrators (Amicon, Beverly, MA).

Pharmacological manipulations. Confluent monolayers of myoepithelial cells were pretreated with tunicamycin (25-200 ng/ml) (Boehringer Mannheim, Indianapolis, IN) for 48 h to study the specific isoforms of CD44 produced by myoepithelial cells. Confluent monolayers of myoepithelial cells (HMS-1-6) and nonmyoepithelial cells (HMECs, C8161, MDA-MB-231, and MDA-MB-468) were also pretreated with PMA (5 μ M) (Sigma Chemical Co.) for 20 min followed by harvesting of the cells 1 to 24 h later. The effects of PMA pretreatment were studied in the presence and absence of plasminogen (10 μ g/ml) (Sigma Chemical Co.) and in the presence of various proteinase inhibitors. The following specific proteinase inhibitors at a 100-fold range of concentrations (the median concentration so designated) were studied: metalloproteinase inhibitors (0.05 mM EDTA, 3.2 nM TIMP-1, and 0.2 mM 1,10 phenanthroline); serine protease inhibitors (10 μ M aprotinin, 0.2 mM leupeptin, 0.2 mM chymostatin and α_1 -antichymotrypsin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 nM plasminogen activator inhibitor-1, and 10 nM α_2 -antiplasmin); and cysteine proteinase inhibitors (1.0 μ M pepstatin A, 10 μ M CA-074, 10 μ M E-64, and 0.2 mM leupeptin). All inhibitors were obtained from Sigma Chemical Co. except for TIMP-1 (Calbiochem-Novabiochem Corp., San Diego, CA) and CA-074 (Peptides International, Louisville, KY).

Antibodies and probes. Isoform-specific monoclonal antibodies to the standard and epithelial isoforms of CD44 were used. Individual and combinations of MAbs to CD44s (IgG₁-clone DF1485, Zymed Laboratories, San Francisco, CA) and CD44v8-10 (Dr. G. J. Dougherty, UCLA, Los Angeles, CA) were used in immunocytochemical, Western blot, and flow cytometric studies. Other monoclonal antibodies used included anti-E-cadherin (Transduction Laboratories, Lexington, KY) and anti- β_1 -integrin (Dr. D. Chang, UCLA). Immunocytochemical studies were performed on formalin-fixed paraffin-embedded sections of human breast tissue according to standard methods. Western blots were performed using the appropriate pri-

mary antibodies at the manufacturers' recommended dilutions and a 1:50,000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL) followed by development of the reaction with the ECL detection system (Amersham Life Sciences). Scion Image software was used for densitometric analysis of bands.

Cellular extractions. Cells were harvested by brief incubation in PBS containing 5 mM EDTA, pelleted, and resuspended in lysis buffer (PBS, 1% (v/v) Nonidet P-40, 5 mM EDTA, and 10 mM PMSF). Lysates were incubated on ice for 10 min and then microcentrifuged for 5 min to pellet nuclei and other insoluble cellular debris. Supernatants were removed and stored at -20°C. Aliquots were thawed, added to an equal volume of nonreducing sample buffer containing 125 mM Tris, 20% (v/v) glycerol, 4.6% (w/v) SDS, pH 6.8, and incubated at 100°C for 5 min. Total cellular proteins were separated on precast 10% Tris-HCl polyacrylamide electrophoresis gels (Bio-Rad Life Science Products, Hercules, CA) and transferred electrophoretically to nitrocellulose membranes (GIBCO-BRL) and subjected to Western blot analysis. Total RNA was extracted using TriZOL (GIBCO-BRL) and 20 μ g of total RNA was loaded/lane in 1.2% agarose gels and Northern blot analyses were performed as previously described [9]. A full-length CD44 cDNA containing the standard CD44 exons (a gift of Dr. G. Dougherty, UCLA) was labeled according to the random primer method of Feinberg and Vogelstein [10]. [α -³²P]dCTP (3000 Ci/mmol) was purchased from Amersham.

Cell fractionations. PMA-pretreated and untreated cells were homogenized in 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM PMSF, 20 μ M leupeptin, 0.15 μ M pepstatin A at 4°C with a Dounce homogenizer and centrifuged at 13,000g for 15 min at 4°C to obtain the particulate fraction. Subcellular fractions of the particulate fraction (P1, nuclear enriched; P2, plasma membrane + mitochondria enriched; and P3, microsomal enriched) were prepared and standard enzyme markers were followed. Further subfractionation of the mitochondria-plasma membrane fraction was carried out by isopycnic centrifugation in Percoll. In some experiments the sedimented membranes were dissolved in SDS sample buffer and assayed for CD44 by Western blot.

Flow cytometric analysis. Myoepithelial cells were pretreated with PMA (5 μ M) for 20 min followed by harvesting of the cells 1 to 24 h later. Cells were harvested with PBS containing 5 mM EDTA and centrifuged. The pellet was then resuspended with FITC-CD44 mAb or control FITC-IgG and incubated on ice for 30 min. Cells were then washed extensively with HBSS containing 0.1% Na azide and 2% fetal bovine serum and subject to flow cytometry using a Becton Dickinson FACScan.

Purification of shed CD44. A confluent monolayer of HMS-1 was pretreated with PMA (5 μ M) for 20 min followed by collection of conditioned medium over the next 24 h. Conditioned medium was collected in basal K-SFM and concentrated up to 10-fold with Centriprep-10 concentrators and subjected to hyaluronan (HA)-affinity chromatography. Purified HA obtained from human umbilical cord was cross-linked to Sepharose 4B. Concentrated HMS-1 conditioned medium (CM) containing shed CD44 was incubated in the affinity column (1 \times 15 cm) at 4°C for 15 h. Controls included bovine serum albumin (BSA) cross-linked to Sepharose 4B or cross-linked Sepharose 4B alone. The unbound fraction from the HA affinity column was washed through with 40 ml of 25 mM Tris, 1.5 mM CaCl₂, 5 mM MgCl₂, and 0.9% NaCl, pH 7.4. The bound fraction was eluted with 0.2 M glycine HCl, pH 3.5, immediately neutralized with 1.0 M Tris/saline and lyophilized. The lyophilized samples were redissolved in medium and used in HA adhesion and migration assays.

HA adhesion and migration assays. A 24-well plate was coated with HA by adding 500 μ l of a solution of potassium hyaluronate purified from human umbilical cord (5 mg/ml in PBS) to each well and incubating the wells overnight at 4°C. Unbound HA was removed by extensive washing with PBS. Nonspecific binding was

blocked by incubating the wells with 10% BSA in PBS for 1 h. The carcinoma cell lines (including the C8161, MDA-MB-231, and MDA-MB-468) and HUVECs (pretreated with bFGF (10 ng/ml)) were separately added to the wells. A total of 2×10^4 cells/well were added in this adhesion assay and incubated for 15 min at 37°C. The effects of HMS-1 CM, anti-CD44, and HA affinity-purified myoepithelial-solubilized CD44 were tested in this adhesion assay by adding these components individually to each well at the time of introducing the cells. Nonadhering cells were removed by a standardized wash procedure. The number of adhering cells was scored using an inverted microscope. Carcinoma cell and endothelial cell migration were separately assayed using a 48-well modified Boyden chamber (Neuro Probe, Inc., Bethesda, MD) with polycarbonate Nucleopore filters of 8 μ m pore size (Nucleopore Corp., Pleasanton, CA). The undersides of these filters were precoated with 1 mg/ml HA. Prior to the migration assays, the filters were washed in PBS and air dried. The lower compartments of the modified Boyden chambers were filled with medium containing 10% fetal calf serum (for the carcinoma cell migration assays) or bFGF (10 ng/ml) for the endothelial cell migration assays. A total of 5×10^4 C8161, MDA-MB-231, MDA-MB-468, or HUVECs were separately added to the upper compartments in this migration assay. Chambers were incubated for 6 h at 37°C. After removal of the filters, cells on the noncoated upper membrane side were gently wiped off. Filters were fixed in methanol, stained with Giemsa solution, and mounted on glass slides. Cells that had migrated to the coated undersides of the filters were counted using high-power fields of a light microscope. The effects of HMS-1 CM, anti-CD44, and HA affinity-purified myoepithelial-solubilized CD44 were tested by adding these components individually to each well at the time of introducing the cells. Both the adhesion and migration assays were performed in quadruplicate. Additional controls in both assays included nonmyoepithelial cell CM, murine IgG₁, and the HA affinity column unbound wash through fraction.

Statistical analysis. Results were analyzed with standard tests of statistical significance, including the two-tailed Student's *t* test and a one-way analysis of variance (ANOVA).

RESULTS

Myoepithelial CD44 Expression

Anti-CD44 immunostaining revealed strong CD44 immunoreactivity in myoepithelial cells surrounding breast ducts and acini (Fig. 1A). All of the myoepithelial cell lines studied (HMS-1–6) expressed both the 85-kDa standard (CD44s) and the 130-kDa epithelial (CD44e) (CD44v8-10) isoforms of CD44. The epithelial isoform was heavily and variably glycosylated producing a smear on Western blot ranging from MW 150–250 kDa. In experiments where HMS cells were pretreated with tunicamycin; however, the heavily glycosylated isoform was reduced to a narrow band of 120–130 kDa, suggesting that it represented the epithelial (CD44v8-10) isoform (Fig. 1B). This fact was confirmed by using epithelial (CD44v8-10) isoform-specific antibodies on Western blot and demonstrating strong signals (data not shown). Epithelial cell lines like HMECs expressed the same CD44 isoforms (the 85-kDa standard (CD44s) and the 130-kDa epithelial (CD44e) (CD44v8-10) isoform) as myoepithelial cells (data not shown).

Myoepithelial-Specific CD44 Shedding

The myoepithelial cell lines studied constitutively shed the 130-kDa epithelial isoform predominantly (Fig. 1C). PMA pretreatment, however, enhanced the shedding of both isoforms ($P < 0.001$) (Fig. 1C). The decrease in cell-associated CD44 and concomitant increase in CD44 in conditioned medium induced by PMA was due to enhanced cell shedding and not a switch in CD44 isoform synthesis from alternative splicing or alterations in levels of CD44 transcripts from changes in gene expression, both of which could contribute to cell surface CD44 alterations (Fig. 1D). Furthermore, the increase in CD44 in conditioned medium never occurred without a concomitant decrease in cell-associated CD44 (Fig. 1C) suggesting that this was a shedding and not a secretory phenomenon. Furthermore, other possibilities to explain the decrease in cell-surface CD44 immunoreactivity such as PMA-induced conformational alterations or PMA-induced alterations in epitope accessibility within the extracellular domain of CD44 were also excluded by these dual observations. Cell fractionation experiments further revealed that virtually all of the CD44 was plasma membrane associated and it was this plasma membrane-associated CD44 that was decreased with PMA pretreatment (Fig. 1E). All of the observations made on Nonidet P-40 detergent extracts of whole cells were confirmed on SDS extracts of purified plasma membranes. These latter studies were necessary because some CD44 activity was retained in the detergent-insoluble pellet of whole cells due to the fact that CD44 is a transmembrane component of partially detergent-insoluble membrane domains, so-called lipid rafts. It was therefore important to use purified plasma membrane preparations to determine what CD44 remained with the plasma membrane and what fraction was truly shed with PMA treatment.

The myoepithelial-cell-associated decrease in CD44 was first in evidence by Western blot 12 h following PMA treatment ($P < 0.001$) (Fig. 2A) but occurred in 2 h by flow cytometric analysis ($P < 0.01$) (Fig. 2B). The PMA-induced reduction in cell-associated CD44 was myoepithelial specific occurring in all of the myoepithelial cell lines examined ($P < 0.001$) but not in any of the nonmyoepithelial cell lines studied ($P > 0.1$) (Figs. 2C, 2D, and 2E). Furthermore, normal HMECs though expressing the same standard and epithelial isoforms of CD44 neither constitutively shed nor shed CD44 in response to PMA (data not shown). Though myoepithelial cells also expressed other adhesion molecules on their surface such as E-cadherin and B1 integrin, they did not shed these molecules constitutively nor in response to PMA ($P > 0.1$) (Fig. 2F). Therefore, the PMA-induced shedding of CD44 was both myoepithelial and CD44 specific. The PMA-induced myoepithelial CD44 shedding was not af-

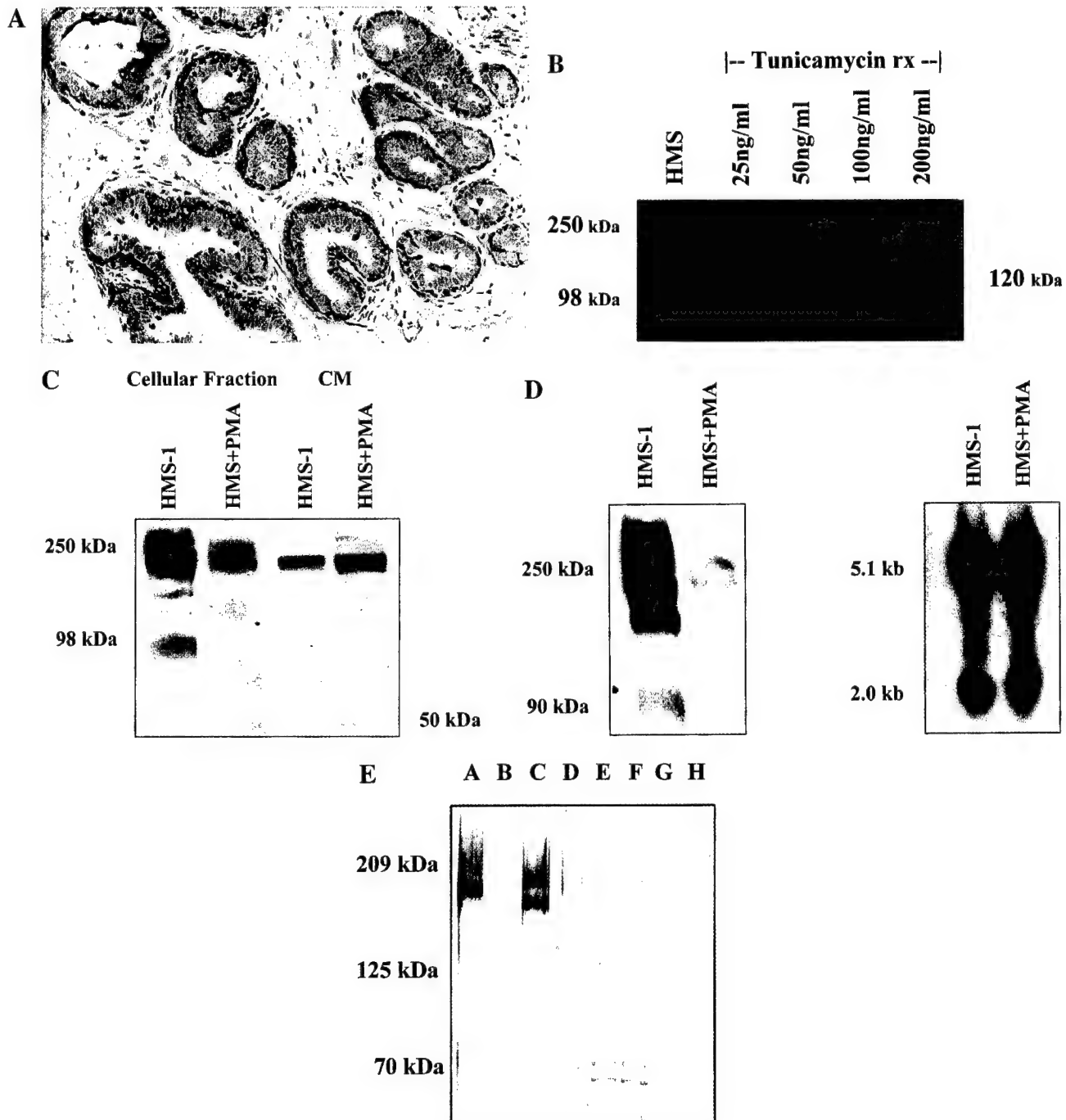


FIG. 1. (A) Anti-CD44 reveals stronger CD44 immunoreactivity in myoepithelial cells surrounding ducts and acini of the breast than in adjacent epithelial cells. In addition to this strong myoepithelial cell CD44 immunoreactivity, scattered CD44 immunoreactivity was also observed at the apical or luminal ends of the ducts and acini (probably reflective of epithelial CD44) as well as within the connective tissue stroma (probably reflective of endothelial cell CD44). CD44 then is a quantitative rather than qualitative marker which distinguishes myoepithelial cells from epithelial and endothelial cells. (B) Tunicamycin at low doses inhibits the synthesis of CD44s and the glycosylation of CD44e, reducing its size to 120–130 kDa and supporting its identity as the epithelial isoform, which was further confirmed by using an epithelial (CD44e) isoform-specific antibody. Tunicamycin at high doses interferes with the synthesis of CD44e as well. (C) Western blot of HMS-1 cellular fractions and CM (concentrated 10-fold) reveals both constitutive and PMA-enhanced CD44 shedding. (D) PMA treatment results in a decrease in cell-associated CD44 (Western blot, left) but no alterations in steady-state mRNA levels nor alternative splicing (Northern blot, right). (E) Cell fractionation experiments with HMS-1 cells reveals that CD44 is present solely in the plasma membrane fraction and the decrease in CD44 following PMA pretreatment is observed in this fraction: lane A, whole cell lysate, no pretreatment; lane B, whole cell lysate, PMA pretreatment; lane C, plasma membrane fraction, no pretreatment; lane D, plasma membrane fraction, PMA pretreatment; lane E, cytosolic fraction, no pretreatment; lane F, cytosolic fraction, PMA pretreatment; lane G, nuclear fraction, no pretreatment; lane H, nuclear fraction, PMA pretreatment.

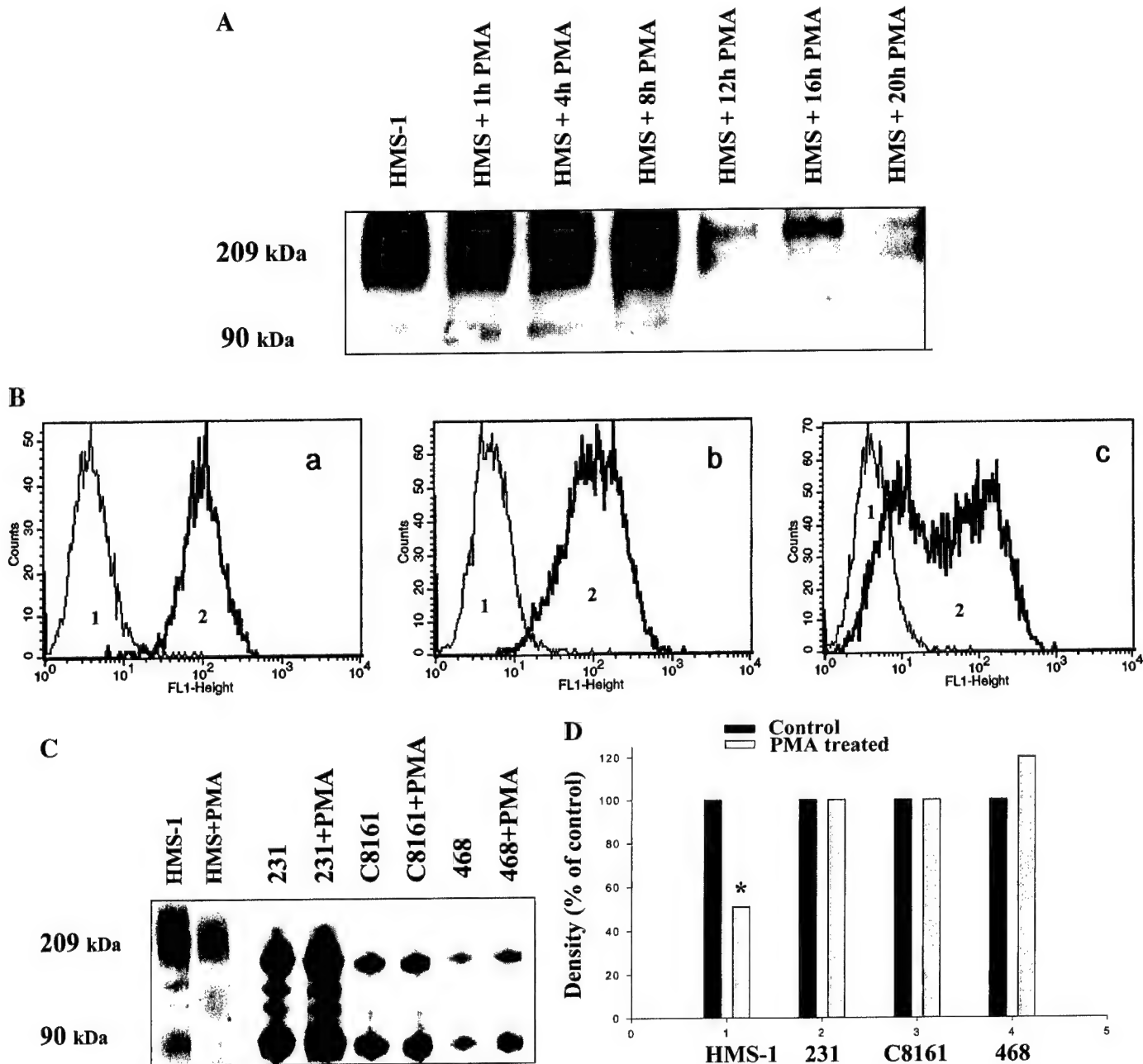


FIG. 2. (A) Time course following 20 min of PMA pretreatment reveals a decrease in cell-associated CD44 at 12 h by Western blot. (B) Flow cytometry reveals a decrease in membrane CD44 at 2 h as demonstrated by an emergence of a cell population with decreased fluorescence. Peak 1, FITC-IgG; Peak 2, FITC-CD44 mAb; a, no treatment; b, PMA pretreatment, harvest at 1 h; c, PMA pretreatment, harvest at 2 h. (C) CD44 shedding induced by PMA is observed only in myoepithelial cells, e.g., HMS-1; other nonmyoepithelial cell lines, MDA-MB-231, C8161, and MDA-MB-468, which express in addition to CD44s alternate isoforms of CD44 (CD44v) not observed in myoepithelial cells, show no shedding of either CD44s or CD44v. (D) Results depicted in (C) are quantitated by densitometric analysis and represent the mean of quadruplicate experiments. CD44 shedding is statistically significant (*) only in myoepithelial cells, e.g., HMS-1. (E) Other myoepithelial cell lines, HMS-3 and HMS-4, exhibit similar shedding. (F) Myoepithelial shedding is limited to CD44; E-cadherin and B1 integrin are not shed. (G) PMA-induced CD44 shedding is inhibited by the chymotrypsin inhibitors chymostatin and α_1 -antichymotrypsin but not at all by plasminogen activator inhibitor-1 (PAI-1) or TIMP-1. Other metallo-, cysteine, and serine proteinase inhibitors also do not inhibit CD44 shedding.

ected by either plasminogen addition (10 μ g/ml) or depletion ($P > 0.1$) (data not shown). The PMA-induced myoepithelial CD44 shedding could be blocked by the

chymotrypsin inhibitors chymostatin and α_1 -antichymotrypsin ($P < 0.01$, $P < 0.01$) but not by general metallo-, cysteine, or other serine proteinase inhibitors ($P > 0.1$)

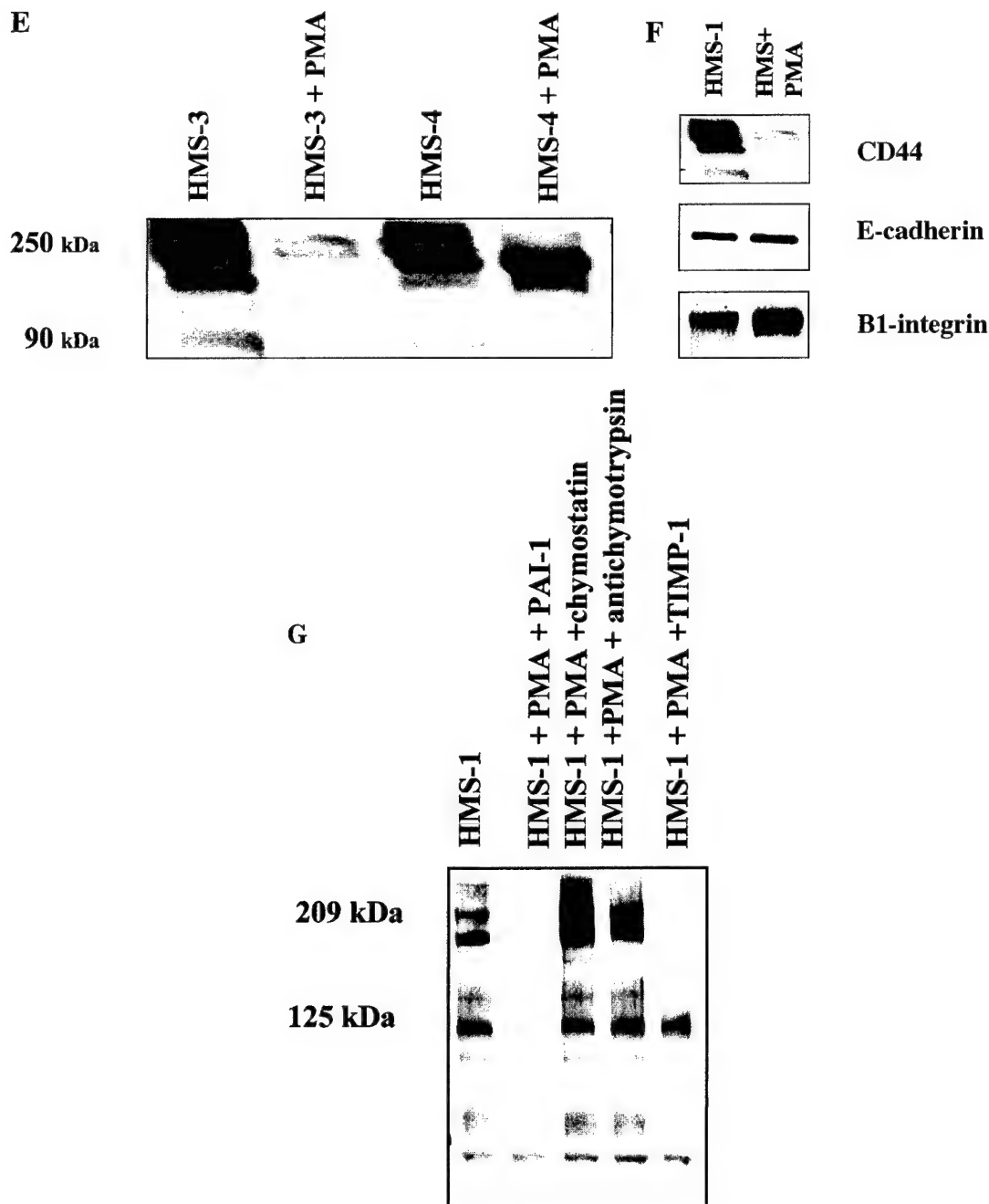


FIG. 2—Continued

(Fig. 2G). These results suggested that the mechanism of CD44 shedding involved proteolytic cleavage by a chymotrypsin or chymotrypsin-like protease.

Myoepithelial CD44 Shedding Effects on Carcinoma and Endothelial HA Adhesion and Migration

Myoepithelial cell CM ($P < 0.05$) and affinity-purified myoepithelial-cell-shed CD44 ($P < 0.01$) were effective at inhibiting both carcinoma cell and endothelial cell adhesion

and migration involving a HA substrate (Figs. 3A and 3B). Anti-CD44 antibodies ($P < 0.01$) were also effective at inhibiting carcinoma cell and endothelial cell adhesion and migration using this same substrate (Figs. 3A and 3B). These results suggest that both carcinoma cells and endothelial cells utilize their membrane CD44 to adhere to HA and that shed or solubilized CD44 from myoepithelial cells can compete for these HA-binding sites and block the ability of carcinoma and endothelial membrane CD44 to effect its

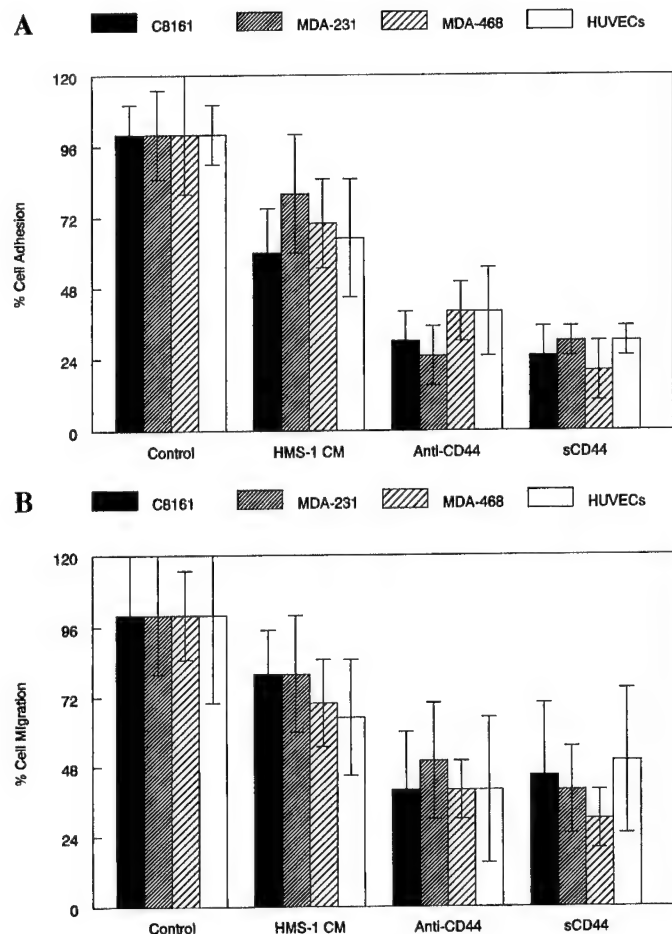


FIG. 3. (A) Effects of HMS-1-conditioned medium (CM) (concentrated 10-fold), anti-CD44 (1/10 dilution), and HA affinity-purified solubilized CD44 (sCD44) (specific binding activity, 5000 ng HA/mg protein) on carcinoma cell (C8161, MDA-MB-231, MDA-MB-468), and HUVEC adhesion on a HA substrate. Baseline cell adhesion for the given cell line is depicted as 100% control and effects of HMS-1 CM, anti-CD44, and purified myoepithelial-solubilized CD44 expressed relative to this control. Results depicted represent means \pm standard errors. (B) Effects of HMS-1-conditioned medium (CM) (concentrated 10 fold), anti-CD44 (1/10 dilution), and HA affinity-purified solubilized CD44 (sCD44) (specific binding activity, 5000 ng HA/mg protein) on carcinoma cell (C8161, MDA-MB-231, MDA-MB-468) and HUVEC migration on a HA substrate. Baseline cell migration for the given cell line is depicted as 100% control and effects of HMS-1 CM, anti-CD44, and purified myoepithelial-solubilized CD44 expressed relative to this control. Results depicted represent means \pm standard errors.

adhesion and haptotaxis functions. In both the adhesion and the migration assays, the addition of nonmyoepithelial cell CM, murine IgG₁, and the HA affinity column unbound washthrough fraction resulted in no differences from control values (data not shown) ($P > 0.1$).

DISCUSSION

Cancer cells come under the influence of important paracrine regulation from the host microenvironment

[11]. Such host regulation may be as great a determinant of a tumor cell's behavior *in vivo* as the specific oncogenic or suppressor alterations occurring within the malignant cell itself and may be mediated by specific extracellular matrix (ECM) molecules, matrix-associated growth factors or host cells themselves [12]. Both positive (fibroblast, myofibroblast, and endothelial cell) and negative (tumor-infiltrating lymphocytes and cytotoxic macrophages) cellular regulators exist that profoundly affect tumor cell behavior *in vivo* [13, 14]. One host cell, the myoepithelial cell, appears to belong to the negative cellular regulator group [1]. Our previous studies have shown that myoepithelial cells and derived cell lines exert multiple suppressive effects on breast carcinoma cells through secretion of a number of different anti-invasive, antiproliferative, and anti-angiogenic molecules [1, 7]. The present study has examined one such molecule, CD44, and specifically its shedding from myoepithelial cells *in vitro* as a potential anti-invasive and antiangiogenic mechanism operating *in vivo*.

CD44 is a cell surface receptor for several ECM components predominant of which is HA but which also include collagen, laminin, fibronectin, and chondroitin sulfate proteoglycan [15]. The binding of HA to CD44 is thought to mediate a number of different biological processes including lymphocytic homing, endothelial chemotaxis (angiogenesis), and tumor cell haptotaxis, invasion and metastasis [16–18]. A number of different regulatory mechanisms exist that can influence the efficacy of CD44–HA binding and the biological processes which are dependent on this interaction. Variant isoforms of CD44 resulting from alternative splicing can influence the affinity of cellular binding [19–24]; phosphorylation of the CD44's cytoplasmic domain can influence the binding properties of its ectodomains [20–24]; and increased shedding or secretion of the CD44 molecule through various mechanisms can either reduce or enhance binding to HA depending on the dynamics of the situation [21–32].

Expression of certain CD44 splice variants are found in several different human cancers. Increased isoform CD44v6 but reduced CD44v9, for example, has been associated with increased metastatic potential [23, 24]. Some studies [25, 26] but not others [27] have implicated specific CD44 isoforms as a prognostic factor in human breast cancer. CD44e (v8–10) is the most common variant isoform in normal epithelial cells and it was this variant that we observed in our myoepithelial cell lines. Significantly greater CD44 immunoreactivity occurred in myoepithelial cells, however. Possible reasons for this included that myoepithelial cells express higher levels of CD44 than their epithelial counterparts or epithelial CD44 is occupied by secreted HA which blocks antibody recognition. Further studies to investigate this latter possibility include preincubating

the sections with hyaluronidase to determine if this changes the pattern of epithelial CD44 staining.

Myoepithelial cells not only express CD44 but shed it constitutively. Since myoepithelial cells rest and synthesize the basement membrane, myoepithelial CD44 may be one adhesion molecule which anchors the myoepithelial cell to the basement membrane. The constitutive shedding of CD44 by myoepithelial cells *in vivo* could therefore participate in normal basement membrane turnover. Our present studies demonstrate that our myoepithelial lines increase their CD44 shedding in response to PMA. This shedding is a true shedding from the plasma membrane and not a secretory phenomenon. The shedding is myoepithelial cell and CD44 specific. Western blot and flow cytometric studies using isoform-specific CD44 antibodies showed that the 130-kDa epithelial isoform was the CD44 isoform that was predominantly shed by myoepithelial cells. Since antibody specificity can rarely be proven to be absolute, and since epitopes are often shared, we, in subsequent studies, have confirmed our findings with PCR using splice-variant-specific primers, given that the entire sequence of the CD44 gene was available. With this more sensitive approach, we have observed that myoepithelial cells also express other CD44 splice variants.

As far as the mechanism of shedding is concerned, increased shedding of ectodomains of molecules such as CD44 could be produced by either extrinsic or intrinsic mechanisms [28]. Hyaluronidase, for example, can digest both HA and the variant isoforms of cell-associated CD44, altering the growth, motility, and metastasizing properties of tumor cells [29]. Alternatively CD44 can be cleaved by intrinsic membrane secretases or sheddases of the metalloproteinase class as has been demonstrated recently in certain glioblastoma, pancreatic, and lung carcinoma cell lines [21, 22]. In these malignant cell lines, increased CD44 shedding resulted in increased haptotaxis and migration because it contributed to an autocrine feedback loop of increased CD44 binding to HA. Spontaneous CD44 shedding from murine lymphocytes has also been observed in the circulation of mice to correlate with immune activity and tumor growth [33]. The CD44 shed in this latter setting seemed intact and functional. Our preliminary studies showing inhibition of CD44 shedding in myoepithelial cells by the chymotrypsin inhibitors chymostatin and α_1 -antichymotrypsin but not by general metallo-, cysteine, or other serine proteinase inhibitors suggest that the mechanism of CD44 shedding in myoepithelial cells involves proteolytic cleavage by a chymotrypsin or chymotrypsin-like protease. We are currently investigating whether this proteolytic mechanism occurring in myoepithelial cells specifically involves a membrane sheddase. The significance of CD44 shedding on myoepithelial cells would be anticipated to be different from the

significance of CD44 shedding on glioblastoma, other malignant cell lines, or lymphocytes since myoepithelial cells are nonmotile and noninvasive [1] and do not migrate even with CD44 shedding. On the other hand soluble CD44 from myoepithelial shedding might be expected to compete with membrane CD44 on carcinoma and endothelial cells for HA-binding sites and our *in vitro* HA adhesion and migration experiments have demonstrated this. Studies by other investigators have also demonstrated that soluble CD44 originating either extrinsically (soluble wild-type CD44-Ig fusion protein) or intrinsically (transfection of cDNAs encoding soluble isoforms of CD44) can compete with tumor cell membrane CD44 for HA-binding sites and exert antitumoral effects including decreased tumorigenicity and increased apoptosis [18, 34, 35]. Since both carcinoma cell invasion and angiogenesis are dependent upon membrane CD44-HA interactions [30-32, 34, 35], myoepithelial-cell-specific shedding of CD44 may reduce the carcinoma and endothelial cell membrane CD44-HA interactions critical to invasion and angiogenesis *in vivo*. Myoepithelial-specific CD44 shedding may therefore contribute to the anti-invasive and antiangiogenic phenotype of myoepithelial cells.

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Myoepithelial-Specific CD44 Shedding Is Mediated by a Putative Chymotrypsin-like Sheddase¹

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Our previous studies have demonstrated that myoepithelial cells, which surround incipient carcinomas *in situ* of the breast and other organs, exert antiinvasive and antiangiogenic effects *in vitro* through the elaboration of a number of different suppressor molecules among which include the shed membrane CD44. The present study addresses the mechanism of this myoepithelial CD44 shedding. This CD44 shedding is enhanced by PMA pretreatment, is specific for myoepithelial CD44, and inhibited by chymotrypsin-like inhibitors (chymostatin, α_1 -antichymotrypsin, TPCK, and SCCA-2) but not by trypsin-like inhibitors (TLCK), nor papain-like inhibitors (SCCA-1) nor hydroxamate-based or general metalloproteinase inhibitors (BB2516 (marimastat), 1,10-phenanthroline, and TIMP-1). The effect of PMA can be mimicked by exogenous chymotrypsin but not by other proteases. The CD44 shedding activity cannot be transferred by conditioned media, cell-cell contact, peripheral membrane, or integral membrane fractions. However, cell-free purified integral plasma membrane fractions obtained from myoepithelial cells pretreated with PMA also exhibit CD44 shedding which is inhibited by chymotrypsin-like inhibitors. These findings support the presence and activation of a putative chymotrypsin-like sheddase as

the mechanism of CD44 shedding in myoepithelial cells. © 2000 Academic Press

Key Words: myoepithelial cells; CD44; chymotrypsin; sheddase; PMA; antiangiogenesis; anti-invasion.

Our previous studies have indicated that myoepithelial cells which surround ductal epithelium of glandular organs such as the breast exert multiple paracrine suppressive effects on incipient cancers which arise from this epithelium (1–3). This paracrine suppression may keep the genetic alterations occurring within malignant epithelial cells in check so that the evolving cancer exists for a number of years only as an *in situ* lesion confined within the ductal system (4). Our laboratory has established immortalized myoepithelial cell lines and transplantable xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3), breast (HMS-4, HMS-5) and bronchus (HMS-6) (with their respective xenografts designated as HMS-#X) (1–3, 5, 6). These cell lines and xenografts express identical myoepithelial markers as normal myoepithelial cells *in situ* and display an essentially normal diploid karyotype. In previous studies we have demonstrated that our myoepithelial cell lines/xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α_1 -antitrypsin, an unidentified 31–33 kDa trypsin inhibitor, thrombospondin-1, soluble bFGF receptors, and maspin (1–3). Our human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion and endothelial cell migration and proliferation (angiogenesis) *in vitro* (3, 7). Our myoepithelial cell lines also inhibit breast carcinoma proliferation *in vitro* through an induction of breast carcinoma cell G₂/M arrest and apoptosis (3), the latter phenomenon of which also occurs *in situ* within DCIS (8). In our previous studies demonstrating that myoepithelial cells inhibited both invasion and

Abbreviations used: HMS, human matrix-secreting; CM, conditioned media; PAI-1, plasminogen activator inhibitor-1; TIMP-1, tissue inhibitor of metalloproteinases; PMA, phorbol 12-myristate 13-acetate; DCIS, ductal carcinoma *in situ*; HMEC, human mammary epithelial cells; K-SFM, keratinocyte-serum-free medium; Mab, monoclonal antibody; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DCI, 3,4-dichloroisocoumarin; TLCK, *N* α -p-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SCCA, squamous cell carcinoma antigen; MMP, matrix metalloproteinase; TNF- α , tumor necrosis factor- α ; TGF α , transforming growth factor- α ; NP-40, Nonidet P-40; PA, plasminogen activator.

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angiogenesis we noted that treatment with PMA potentiated both types of suppression (1, 7). In examining possible mechanisms for these effects we surveyed our myoepithelial cell lines for PMA-induced changes in expression levels of general categories of molecules that had been implicated in tumor invasion and angiogenesis, namely proteinases/proteinase inhibitors, angiogenic factors/angiogenic inhibitors and adhesion molecules. In the latter category we noted in a recent study (9) that PMA profoundly decreased the levels of surface CD44 on myoepithelial cells through a shedding phenomenon. In this study (9), we demonstrated that myoepithelial cells constitutively express and shed both the 85 kDa standard (CD44s) and the 130 kDa epithelial (CD44v8-10) isoforms and that PMA pretreatment enhances this shedding. Our observations were made by using CD44 isoform-specific antibodies as well as CD44 slice-variant-specific primers (9). The decrease in cell-associated CD44 and concomitant increase in CD44 in conditioned media (CM) was due to PMA-enhanced cell shedding and not altered levels of CD44 synthesis from either alternative splicing or changes in gene transcription, either of which could contribute to cell surface CD44 alterations. Furthermore the increase in CD44 in CM never occurred without a concomitant decrease in cell-associated CD44 suggesting that this was a true shedding and not a secretory phenomenon. Furthermore other possibilities to explain the decrease in cell-surface CD44 immunoreactivity such as PMA-induced conformational alterations or PMA-induced alterations in epitope accessibility within the extracellular domain of CD44 were also excluded by these dual observations. Cell fractionation experiments further revealed that virtually all of the CD44 was plasma membrane associated and it was this membrane-associated CD44 that was decreased with PMA pretreatment. The myoepithelial cell-associated decrease in CD44 was first in evidence by Western blot 12 h following PMA treatment but was detected within 2 h by flow cytometric analysis. The PMA-induced reduction in cell-associated CD44 was observed in all 6 of the myoepithelial cell lines examined but not in any of the nonmyoepithelial lines. Furthermore normal mammary epithelial cells (HMECs) though expressing the same standard and variant (epithelial) isoforms of CD44 as myoepithelial cells did not shed CD44 in response to PMA. Because the CD44 shedding phenomenon seemed to be myoepithelial specific, we decided to study it further and investigate its mechanism in the present study.

MATERIALS AND METHODS

Cell lines. Human myoepithelial cell lines (HMS-1-6) from benign myoepithelial tumors of diverse origins had been previously established in our laboratory (1-3, 5, 6) and maintained in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50 μ g/ml) and recombinant human epidermal growth

factor (5 ng/ml). (GIBCO-BRL, Gaithersburg, MD). Other nonmyoepithelial cell lines used included Hs578T (American Type Culture Collection (ATCC), Rockville, MD), and human squamous cell carcinoma lines of salivary gland and vulva, A253 and A431 (ATCC). These latter cell lines were maintained in Eagle MEM supplemented with 10% FBS (GIBCO-BRL) and penicillin-streptomycin antibiotics. Conditioned media from the myoepithelial cell lines untreated and pretreated with PMA (5 μ M) was collected in basal K-SFM and concentrated up to 10-fold with Centrprep-10 concentrators (Amicon, Beverly, MA). Coculture experiments involving cell-cell contact of PMA-pretreated and untreated cell types of various combinations were also conducted.

Pharmacological manipulations. Confluent monolayers of myoepithelial cells (HMS-1-6) were also pretreated with PMA (5 μ M) (Sigma Chemical Co., St. Louis, MO) for 20 min followed by harvesting of the cells 1 to 24 h later. The effects of PMA pretreatment were studied in the presence and absence of plasminogen (10 μ g/ml) and in the presence of various proteinase inhibitors. The following specific proteinase inhibitors at a 10- to 1000-fold range of concentrations (the median concentration so designated) were studied: hydroxamate-based and general metalloproteinase inhibitors including 0.05 mM EDTA, 100 μ M TAPI, 3.2 nM TIMP-1, 0.2 mM 1,10 phenanthroline and 100 μ M BB2516 (marimastat); trypsin-like, chymotrypsin-like and general serine protease inhibitors including 10 μ M aprotinin, 0.2 mM leupeptin, 0.2 mM chymostatin, 0.2 mM α_1 -antichymotrypsin, 10 nM plasminogen activator inhibitor-1, 10 nM α_2 -antiplasmin, 100 μ M 3,4 DCI, 540 nM TLCK, 100 μ M TPCK and 5 μ M SCCA-2; and papain-like and general cysteine proteinase inhibitors including 1.0 μ M pepstatin A, 10 μ M CA-074, 10 μ M E-64, 10 μ M steffin A, 5 μ M SCCA-1, 0.2 mM leupeptin and 0.2 mM cystatin. All inhibitors were obtained from Sigma Chemical Co. except for TIMP-1 (Calbiochem-Novabiochem Corp., San Diego, CA), CA-074 (Peptides International, Louisville, KY), BB2516 (a gift of Dr. Howard Reber, UCLA, Los Angeles, CA), SCCA-1 and SCCA-2 (gifts of Dr. Gary Silverman, Children's Hospital, Boston, MA) and TAPI (Immunex Corp., Seattle, WA). Myoepithelial cells (HMS-1-6) and nonmyoepithelial cells (Hs578T, A253, and A431) were also treated with different proteinases. The following proteinases at a 10- to 1000-fold range of concentrations (the median concentration so designated) were used: type I collagenase (human MMP-1) (0.05 mU) (Oncogen, Cambridge, MA), chymotrypsin, trypsin, elastase, pronase (1 U each) (Sigma Chemical Co.), hyaluronidase (1 U) (Sigma Chemical Co.) and cathepsins B (1 U), D (1 U), L (0.5 mU), and G (2.0 mU) (all from Calbiochem, La Jolla, CA).

Antibodies. Monoclonal antibodies to the standard and epithelial isoforms of CD44 were used. Individual and a combination of MAb to CD44s (IgG1-clone DF1485, Zymed Laboratories, San Francisco, CA) and CD44v8-10 (Dr. Graeme J. Dougherty, UCLA, Los Angeles, CA) were used in Western blot and flow cytometric studies. Western blots were performed using the appropriate primary antibodies at the manufacturers' recommended dilutions and a 1:50,000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL) followed by development of the reaction with the ECL detection system (Amersham Life Sciences, Arlington Heights, IL). A loading control antibody to β -actin (a gift of Dr. Howard Reber, UCLA) was used in all immunoblotting experiments to normalize for cell protein. Scion Image software was used for densitometric analysis of bands. Cells were harvested by brief incubation in PBS containing 5 mM EDTA, pelleted and resuspended in lysis buffer A (PBS, 1% (v/v) NP-40, 5 mM EDTA and 10 mM PMSF). Lysates were incubated on ice for 10 min, then microcentrifuged for 5 min to pellet nuclei and other insoluble cellular debris. Supernatants were removed and stored at -20°C . Aliquots were thawed, added to an equal volume of non-reducing sample buffer containing 125 mM Tris, 20% (v/v) glycerol, 4.6% (w/v) SDS, pH 6.8, and incubated at 100°C for 5 min. Total cellular proteins were separated on precast 10% Tris-HCl polyacrylamide electrophoresis gels (Bio-Rad Life Science Products, Hercules,

CA), and transferred electrophoretically to nitrocellulose membranes (Gibco BRL, Gaithersburg, MD) and subjected to Western blot analysis.

Flow cytometric analysis. Myoepithelial cells were pretreated with PMA (5 μ M) for 20 min followed by harvesting of the cells 1 to 24 h later. Cells were harvested with PBS containing 5 mM EDTA, and centrifuged. The pellet was then resuspended with FITC-CD44 mAb or control FITC-IgG and incubated on ice for 30 min. Cells were then washed extensively with HBSS containing 0.1% Na azide and 2% fetal bovine serum and subject to flow cytometry using a Becton-Dickinson FACScan.

Cell fractionations. PMA-pretreated cells were homogenized in buffer B (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM PMSF, 20 μ M leupeptin, 0.15 μ M pepstatin A) at 4°C with a Dounce homogenizer and centrifuged at 13,000g for 15 min at 4°C to obtain the particulate fraction. Subcellular fractions of the particulate fraction (P1, nuclear enriched; P2, plasma membrane + mitochondria enriched; and P3, microsomal enriched) were prepared and recommended enzyme markers were followed as described previously (10). Further subfractionation of the mitochondria-plasma membrane fraction was carried out by isopycnic centrifugation in Percoll. In some experiments the sedimented membranes were dissolved in SDS sample buffer and assayed for CD44 by Western blot. In other experiments the sedimented membranes were subsequently extracted in 1 M NaCl/EDTA (peripheral fraction) or detergent 1% NP-40 (integral fraction) and transferred to a layer of untreated HMS-1 cells to study the effect of this transfer on CD44 shedding. In other experiments the sedimented membranes were suspended in ice-cold Tris-HCl (30 mM, pH 7.2) containing various components to be tested. The reaction mixture (20 μ l) was transferred to 37°C and incubated for 1–12 h. The reaction was stopped by the addition of an equal quantity of SDS sample buffer and incubated at 100°C for 5 min. Then aliquots of reaction mixtures were subjected to Western blot analysis using CD44 mAb. The sedimented membranes were also added to 10 volumes of ice-cold 1 M NaCl in 30 mM Tris-HCl (pH 7.2) and kept 15 min on ice. Membranes were collected by centrifugation at 10,000g for 15 min at 4°C. Washed membranes were reconstituted in ice-cold Tris-HCl (30 mM, pH 7.2) and incubated and processed as before.

Statistical analysis. Results were analyzed with standard tests of statistical significance, including the two-tailed Student's *t* test and a one-way analysis of variance (ANOVA).

RESULTS

Mechanism of myoepithelial CD44 shedding. The myoepithelial cell-associated decrease in CD44 was detected within 2 h by flow cytometric analysis following PMA pretreatment ($P < 0.01$) (Fig. 1A). The PMA-induced myoepithelial CD44 shedding was not affected by either plasminogen addition (10 μ g/ml) or depletion ($P > 0.1$) or α_2 -antiplasmin (Fig. 1B), indicating that the shedding was not mediated by plasminogen activators (PA) or plasmin. The PMA-induced myoepithelial CD44 shedding could be blocked however by the chymotrypsin-like serine proteinase inhibitors, chymostatin, α_1 -antichymotrypsin, TPCK and SCCA-2 (Fig. 1C) ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.05$) but not by trypsin-like serine proteinase inhibitors like plasminogen activator inhibitor (PAI-1) ($P > 0.1$), papain-like cysteine proteinase inhibitors ($P > 0.1$), or hydroxamate-based or general metalloproteinase inhibitors like TIMP-1 (Fig. 1C) ($P > 0.1$) (Table I). With

the chymotrypsin-like proteinase inhibitors, e.g., chymostatin, there was a dose response of inhibition (Fig. 1D). The chymotrypsin-like inhibitors alone in the absence of PMA-pretreatment exerted no effect on myoepithelial CD44. These results implicated a putative chymotrypsin-like sheddase. As further indirect proof, purified exogenous chymotrypsin mimicked the effect of PMA. Chymotrypsin cleaved CD44 on myoepithelial cells over a similar time course of 12 h (Fig. 1E). Chymostatin inhibited the cleavage of CD44 by chymotrypsin. The inhibitory effects of chymostatin and other chymotrypsin-like serine proteinase inhibitors like SCCA-2 (but not the papain-like cysteine proteinase inhibitors like SCCA-1 and cystatin) on both exogenous CD44 cleavage and on PMA-induced myoepithelial CD44 shedding were also observed in flow cytometric studies (Fig. 1F).

Evidence for a specific myoepithelial sheddase. The CD44 shedding activity could not be transferred by CM (Fig. 2A) nor abolished by the removal of CM (Fig. 2B). The susceptibility of myoepithelial CD44 to exogenous proteolytic cleavage was highly chymotrypsin sensitive and relatively insensitive to other proteases (Fig. 2C). Other nonmyoepithelial CD44 did not exhibit this chymotrypsin sensitivity (Fig. 2D). These other nonmyoepithelial CD44 also did not exhibit PMA-induced shedding (data not shown). Coculture experiments where PMA-pretreated HMS-1 cells were subsequently mixed with untreated HMS-1 cells at dilutions to insure cell-cell contact between each of the populations showed no evidence of CD44 shedding in the untreated cells; furthermore when peripheral membrane fractions or integral membrane fractions of PMA-pretreated HMS-1 cells were transferred to untreated HMS-1 cells there was no evidence of CD44 shedding in the HMS-1 cells (data not shown). However cell-free membrane preparations of PMA-pretreated HMS-1 cells showed a progressive CD44 shedding over 1–8 h (Fig. 2E). This CD44 shedding was equally observed in membranes that had been washed with 1 M NaCl in 30 mM Tris-HCl (pH 7.2) to remove peripheral membrane proteins suggesting that the factor responsible for the CD44 shedding was an integral membrane protein. As before, this CD44 shedding could be inhibited by the chymotrypsin-like serine proteinase inhibitors but not by the trypsin-like serine proteinase inhibitors, the papain-like cysteine proteinase inhibitors or the metalloproteinase inhibitors. CD44 shedding was not observed in membrane preparations obtained from HMS-1 cells that were initially untreated or subsequently treated with proteinase inhibitors alone. The collective findings suggest that the membrane factor responsible for shedding and its substrate, CD44, both have to be present in *cis* orientation for activity and support the presence and activation of a putative

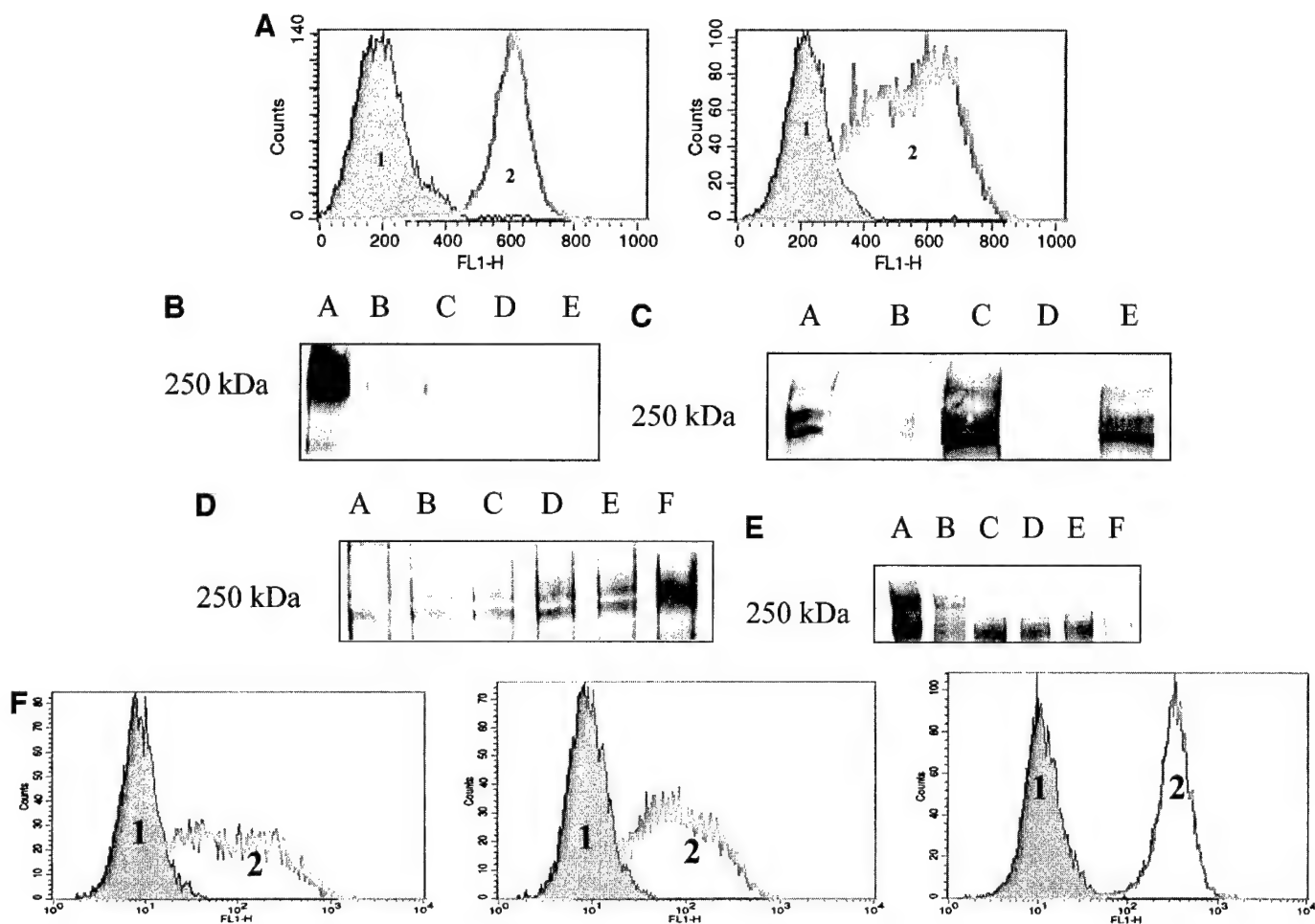


FIG. 1. CD44 flow cytometry or Western blot of HMS-1 cells: (A) Flow cytometry of untreated HMS-1 cells depicts cell surface CD44 fluorescence (top). PMA pretreatment results in a decrease in cell surface CD44 at 2 h as demonstrated by an emergence of a cell population with decreased fluorescence (bottom). Peak 1, FITC-IgG; peak 2, FITC-CD44 mAb; top, no pretreatment; bottom, PMA pretreatment. (B) PMA-induced CD44 shedding is not affected by either the presence or absence of plasminogen or the presence of α_2 antiplasmin: Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1; lane C, PMA-pretreated HMS-1 cells in the absence of plasminogen; lane D, PMA-pretreated HMS-1 cells in the presence of plasminogen; lane E, PMA-treated cells in the presence of α_2 antiplasmin. (C) PMA-induced CD44 shedding is inhibited by chymostatin and α_1 -antichymotrypsin but not at all by plasminogen activator inhibitor-1 (PAI-1) or TIMP-1. Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1 in the presence of PAI-1; lane C, PMA-pretreated HMS-1 in the presence of chymostatin; D, PMA-pretreated HMS-1 cells in the presence of TIMP-1; E, PMA-pretreated HMS-1 cells in the presence of α_1 -antichymotrypsin. Other chymotrypsin-like serine proteinase inhibitors, TPCK, and SCCA-2 (Table I) were effective at inhibition but not inhibitors belonging to other classes. (D) Dose response of increasing concentrations of chymostatin on CD44 shedding inhibition: Lane A, PMA-pretreated HMS-1 cells in the presence of 2 μ M chymostatin; lane B, PMA-pretreated HMS-1 in the presence of 10 μ M chymostatin; lane C, PMA-pretreated HMS-1 in the presence of 20 μ M chymostatin; lane D, PMA-pretreated HMS-1 in the presence of 200 μ M chymostatin; lane E, PMA-pretreated HMS-1 in the presence of 1 mM chymostatin; lane F, PMA-pretreated HMS-1 in the presence of 2 mM chymostatin. Other chymotrypsin-like inhibitors showed a similar dose response. (E) Time course of exogenous chymotrypsin (1 unit) cleavage of HMS-1 CD44. Lane A, untreated HMS-1 cells; lane B, HMS-1 cells treated with chymotrypsin (1 unit) for 1 h; lane C, HMS-1 cells treated with chymotrypsin (1 unit) for 2 h; lane D, HMS-1 cells treated with chymotrypsin (1 unit) for 4 h; lane E, HMS-1 cells treated with chymotrypsin (1 unit) for 8 h; lane F, HMS-1 cells treated with chymotrypsin (1 unit) for 12 h. (F) Flow cytometry of PMA-pretreated HMS-1 cells in the presence of cystatin, top; PMA-pretreated HMS-1 cells in the presence of SCCA-1, middle; PMA-pretreated HMS-1 cells in the presence of SCCA-2, bottom. Only SCCA-2 inhibited the CD44 shedding. Peak 1, FITC-IgG; Peak 2, FITC-CD44 mAb.

chymotrypsin-like sheddase as the mechanism of PMA-induced CD44 shedding in myoepithelial cells.

DISCUSSION

Cancer cells come under the influence of important paracrine regulation from the host microenvironment

(11). Both positive (fibroblast, myofibroblast and endothelial cell) and negative (tumor infiltrating lymphocytes and cytotoxic macrophages) cellular regulators exist that profoundly affect tumor cell behavior *in vivo* (12–14). One host cell, the myoepithelial cell, appears to belong to the negative cellular regulator group (1). Our previous studies have shown that myoepithelial

TABLE I

Representative Inhibitory Spectrum of the Putative Myoepithelial CD44 Sheddase

Proteinase	Inhibitory class	Activity
EDTA	M	-----
TIMP-1	M	-----
1,10-Phenanthroline	M	-----
BB2516	M	-----
TAPI	M	-----
PAI-1	S	-----
3,4 DCI	S	-----
TLCK	S	-----
TPCK	S	+++
SCCA-2	S	+++
α -1-Antichymotrypsin	S	+++
Steffin A	C	-----
SCCA-1	C	-----
Chymostatin	S, C	++++
CA-074	C	-----
E-64	C	-----
Cystatin	C	-----

Note. M, metalloproteinase inhibitor; S, serine proteinase inhibitor; C, cysteine proteinase inhibitor.

cells and derived cell lines exert multiple suppressive effects on carcinoma cells through secretion of a number of different anti-invasive, antiproliferative, and antiangiogenic molecules (1, 7). Another candidate paracrine suppressor molecules is shed CD44. The present study has examined the mechanism of myoepithelial CD44 shedding.

CD44 is a cell surface receptor for several extracellular matrix components predominant of which is hyaluronan but which also include collagen, laminin, fibronectin and chondroitin sulfate proteoglycan (15). The binding of hyaluronan to CD44 is thought to mediate a number of different biological processes including lymphocytic homing, endothelial chemotaxis (angiogenesis) and tumor cell haptotaxis, invasion and metastasis (16–18). A number of different regulatory mechanisms exist which can influence the efficacy of CD44–hyaluronan binding and the biological processes which are dependent on this interaction. Variant isoforms of CD44 resulting from alternative splicing can influence the affinity of cellular binding (19–24); phosphorylation of the CD44's cytoplasmic domain can influence the binding properties of its ectodomains (20–24); and increased shedding or secretion of the CD44 molecule through various mechanisms can either reduce or enhance binding to hyaluronan depending on the specifics and the dynamics of the situation (21–29).

Our present studies demonstrate that myoepithelial cells shed CD44. The shedding is myoepithelial cell CD44 specific. Increased shedding of ectodomains of molecules such as CD44 can be produced by either extrinsic or intrinsic mechanisms (21, 22, 25–28). Hy-

aluronidase, for example, can digest both hyaluronan and the variant isoforms of cell-associated CD44, altering the growth, motility and metastasizing properties of tumor cells (25, 26). Alternately CD44 can be cleaved by intrinsic membrane secretases or sheddases (21, 22).

Our studies addressing the mechanism of the PMA-induced CD44 shedding in myoepithelial cells indicate that a putative chymotrypsin-like sheddase is involved. There has been a recent interest in membrane sheddases or membrane convertases as they are sometimes designated (30–41). Membrane sheddases have been implicated in the shedding of a number of different membrane and cell surface molecules which include a diverse range of membrane proteins of Type I or Type II topologies (31). Examples of molecules shed by sheddase mechanisms include Alzheimer's amyloid precursor protein, angiotensin converting enzyme, TGF- α , the tumor necrosis ligand and receptor super-families (33) and cell adhesion molecules such as L-selectin (38, 39) and CD44 (21, 22, 36, 37). Most of the sheddases identified to date have been metalloproteinases but not necessarily matrix metalloproteinases (33, 38, 39). The identification of these sheddases has rested mainly on indirect evidence as ours has, which is based on the specificities of a broad spectrum of proteinase inhibitors. The vast majority of putative sheddases have not been purified or cloned. The one exception has been tumor necrosis factor- α converting enzyme (TACE), a metalloproteinase-disintegrin sheddase, demonstrated to have catalytic function, and thought responsible for the shedding of diverse cell surface proteins including, in addition to TNF- α , TGF α and L-selectin but interestingly not CD44 (35). CD44 has been reported to be cleaved and shed, on the other hand, by stimulated human granulocytes and certain malignant cell lines including glioblastoma lines. Both cellular sheddings were induced by PMA and inhibited by metalloproteinase inhibitors including TIMP-1 and 1,10-phenanthroline (21, 22, 36, 37). In our studies with myoepithelial cells, diverse metalloproteinase inhibitors including TIMP-1, 1,10-phenanthroline, EDTA, and hydroxamate-based inhibitors, TAPI and BB2516 were not effective in inhibiting PMA-induced CD44 shedding; rather diverse chymotrypsin-like serine proteinase inhibitors but not trypsin-like serine or papain-like cysteine proteinase inhibitors were effective. When we first began investigating the inhibitory spectrum of our putative sheddase, we began with general inhibitors of each proteinase class. We initially noted that chymostatin exhibited the greatest inhibitory activity. Because this proteinase inhibitor had general inhibitory activity against both chymotrypsin-like serine proteinases as well as cysteine proteases, we further defined the inhibitory spectrum of our putative sheddase by investigating two additional proteinase inhibitors: cystatin and α ₁-antichymotrypsin.

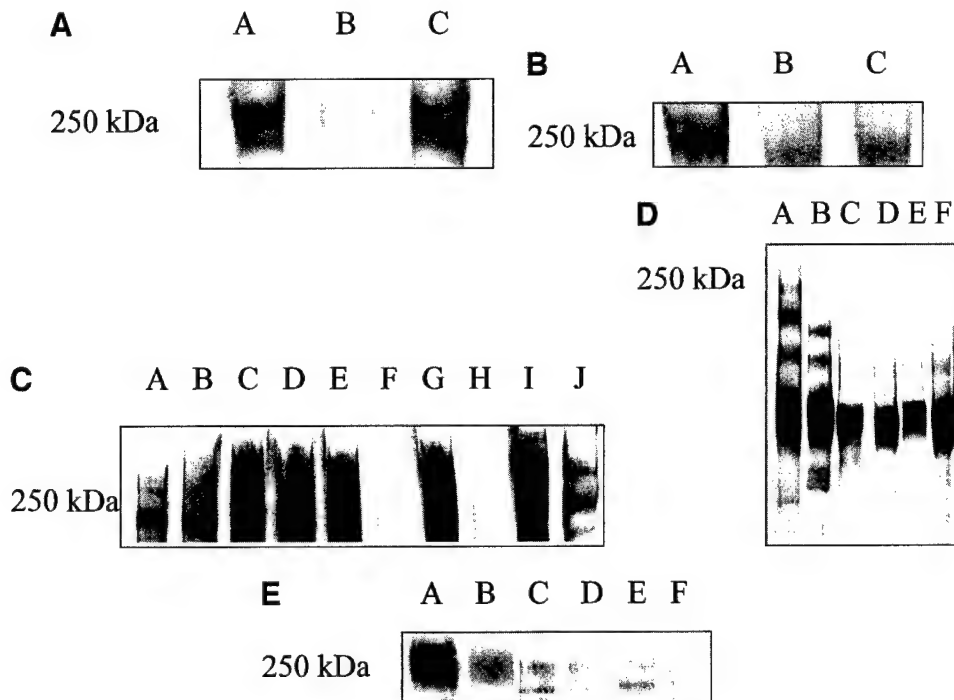


FIG. 2. Western blots of HMS-1 fractions: (A) HMS-1 CD44 sheds following PMA pretreatment but this activity cannot be transferred in CM: Lane A, untreated HMS-1 cell; lane B, PMA-pretreated HMS-1 cells; lane C, CM from HMS-1 cells pretreated with PMA and transferred to untreated HMS-1 cells followed by the usual cellular harvest. (B) HMS-1 CD44 sheds following PMA pretreatment but this activity can not be abolished by hourly removal of CM over the ensuing 24 h from HMS-1 cells following PMA-pretreatment. Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1 cells; lane C, HMS-1 cells pretreated with PMA whose CM was removed hourly for 24 h followed by the usual cellular harvest. (C) Susceptibility of myoepithelial CD44 to different proteases: Cathepsin G (lane A); cathepsin L (lane B); cathepsin D (lane C); cathepsin B (lane D); trypsin (lane E); chymotrypsin (lane F); hyaluronidase (lane G); elastase (lane H); MMP-1 (lane I); pronase (lane J). No enzyme other than chymotrypsin and elastase cleaved myoepithelial CD44. (D) Exogenous chymotrypsin CD44 cleavage is specific for myoepithelial cells: Lane A, untreated Hs578T cells; lane B, chymotrypsin-treated Hs578T cells; lane C, untreated A253 cells; lane D, chymotrypsin-treated A253 cells; lane E, untreated A431 cells; lane F, chymotrypsin-treated A431 cells. Although these nonmyoepithelial cells express alternate forms of CD44, their CD44 does not exhibit chymotrypsin cleavage. (E) CD44 shedding in membrane preparations of PMA-pretreated HMS-1 cells. A progressive decrease in CD44 is observed over time: Lane A, no pretreatment; lane B, PMA pretreatment followed by 1 h incubation; lane C, PMA pretreatment followed by 2 h incubation; lane D, PMA pretreatment followed by 3 h incubation; lane E, PMA pretreatment followed by 4 h incubation; lane F, PMA pretreatment followed by 8 h incubation.

The former had inhibitory activity against papain-like cysteine proteases where the latter had inhibitory activity against serine proteinase inhibitors. We observed inhibitory activity only with α_1 -antichymotrypsin. Then we compared SCCA-2 with SCCA-1. These latter inhibitors are proteinase inhibitors that map to a serpin cluster at 18q21.3 but differ in inhibitory spectrum (42, 43). SCCA-1 inhibits only papain-like cysteine proteases, whereas SCC2-A inhibits chymotrypsin-like serine proteinases but interestingly not purified chymotrypsin (42, 43). Yet SCCA-2 was active against our putative myoepithelial sheddase. Finally we compared TPCK, an inhibitor of chymotrypsin-like serine proteinases with TLCK, an inhibitor of trypsin-like serine proteinases and found that only TPCK was active against our putative myoepithelial sheddase. These four separate lines of evidence, although indirect, all point to a putative chymotrypsin-like sheddase.

From our studies, three requirements were necessary for CD44 shedding to occur (1): The substrate, CD44, had to be susceptible to cleavage in the first place. The susceptibility of a particular isoform of CD44 to cleavage may not necessarily be a function of its primary amino acid sequence determined by its specific splice variation but rather a function of its tertiary structure and/or degree of glycosylation. CD44 molecules on nonmyoepithelial cells were not susceptible to cleavage by exogenous purified chymotrypsin suggesting that their CD44 might not be susceptible to shedding from an endogenous chymotrypsin-like sheddase even if one existed in those cells (2). There must be activation of the sheddase. In our past studies, we have shown that PMA activates and causes membrane association of protein kinase C (44). Presumably this association activates the sheddase responsible for CD44 shedding. The mechanism of activation remains unknown but could be due to direct activation of the

sheddase by phosphorylation, increased access to the substrate as a result of phosphorylation, or conformational modification of the substrate itself making it susceptible to cleavage. From our studies we can not yet determine whether nonmyoepithelial cell lines lack the sheddase or lack the activating response to PMA or both since their CD44 does not appear to be susceptible to chymotrypsin cleavage in the first place (3). Even if the CD44 is of the type which is susceptible to cleavage and even if the membrane sheddase exists and can be activated, both sheddase and CD44 substrate must be in *cis* orientation. Actually this requirement is confirming evidence that our putative chymotrypsin-like activity is indeed a sheddase. Since the sheddase is not secreted, transfer of activity or abolishment of activity by removal of CM could not be achieved. Transfer of sheddase activity likewise could not be achieved by cell-cell contact or membrane extracts because both produce a *trans* orientation.

Our observations that myoepithelial cell CD44 is equally susceptible to both exogenous chymotrypsin cleavage as well as endogenous PMA-induced shedding is again indirect evidence that we are dealing with a chymotrypsin-like sheddase. Certainly we do not know whether both exogenous and endogenous molecules cleave the same sites of CD44, only that CD44 is susceptible to cleavage by both molecules. Obviously the exogenous chymotrypsin is not restricted by the *cis* orientation requirement.

The significance of CD44 shedding from myoepithelial cells would be anticipated to have paracrine tumor suppressive effects on both carcinoma cells themselves as well as on endothelial cells (angiogenesis) from recent studies (45, 46). These studies have demonstrated that soluble CD44 can have autocrine suppressive effects on tumor cells: soluble CD44 originating either extrinsically (soluble wild-type CD44-Ig fusion protein) or intrinsically (transfection of cDNAs encoding soluble isoforms of CD44) can compete with tumor cell membrane CD44 for hyaluronan binding sites and exert antitumoral effects including decreased tumorigenicity and increased apoptosis (18, 45, 46). Since both carcinoma cell invasion and angiogenesis are dependent upon membrane CD44-hyaluronan interactions (27–29, 45, 46), myoepithelial cell specific shedding of CD44 could reduce the carcinoma and endothelial cell CD44-hyaluronan interactions critical to invasion and angiogenesis *in vivo*. CD44 shedding from myoepithelial cells would therefore be anticipated to have paracrine suppressive effects on tumors.

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Fiberoptic Ductoscopy for Patients with Nipple Discharge

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BACKGROUND. Breast carcinoma and precancer are thought to start in the lining of the milk duct or lobule, yet until recently, we have not had direct access to this area other than by blindly removing tissue by core biopsy or fine-needle aspiration. Fiberoptic ductoscopy (FDS) is an emerging technique allowing direct visual access to the ductal system of the breast through nipple orifice exploration.

METHODS. We applied ductoscopy to 259 women who had nipple discharge, and we analyzed the visual findings, the cytological washings, and the subsequent histopathology.

RESULTS. In 92 (36%) of these women, fiberoptic ductoscopy was successful in detecting an intraductal papillary lesion. Of these observed lesions, 68 (74%) were single papilloma, 21 (23%) were multiple discrete papillomas, and 3 (3%) were diffuse intraductal thickening which corresponded to diffuse papillomatosis on histopathological analysis. The overall positive predictive value of FDS screening was 83%. Of the lesions observed, 29.8% were located in the main (segmental) duct, 43.9% lesions in the first branch, 17.5% lesions in the second branch, 7.9% in the third branch, and 0.9% in the fourth branch. These lesions had an overall average distance of 2.7 cm from the nipple orifice. Ductal washings performed at the time of ductoscopy were effective at obtaining representative exfoliated ductal cells which could be evaluated for the presence of clumps (> 50 cells), clumps with atypia or single ductal cells. The presence of clumps with positive FDS increased the positive predictive value to 86%.

CONCLUSIONS. Fiberoptic ductoscopy currently offers a safe alternative to ductography in guiding subsequent breast surgery in the treatment of nipple discharge. *Cancer* 2000;89:1512-9. © 2000 American Cancer Society.

KEYWORDS: nipple discharge, intraductal papillary lesions, fiberoptic ductoscopy.

Breast cancer and precancer are thought to start in the lining of the milk duct or lobule, yet until recently, we have not had direct access to this area other than by blindly removing tissue by core biopsy or fine-needle aspiration. Fiberoptic ductoscopy is an emerging technique allowing direct visual access to the ductal system of the breast through nipple orifice cannulation and exploration.¹

Spontaneous nipple discharge is a common health problem reported to occur at least intermittently in up to 10% of women who undergo routine health examinations.² In this article, discharge refers to any type of discharge which can be serous, serosanguineous, milky, watery, or bloody. Common causes of these various types of nipple discharge are intraductal papilloma or papillomatosis, which are observed in 35% to 48% of cases based on surgical pathology analysis of excised tissues.³ Because of this, surgery is indicated when the diagnosis is suspected.² Because many cases of nipple discharge occur, however, without the presence of an intraductal lesion, breast duc-

tography has been a technique employed in the United States to indicate the presence and location of a site of intraductal obstruction thought to correspond to the presence of intraductal papilloma. Breast ductoscopy, however, is a technique which historically has not enjoyed popularity in the United States. Whereas early attempts at ductoscopy used a rigid ductoscope, fiberoptic ductoscopy is now an emerging technique allowing direct visual access to the ductal system of the breast through nipple orifice cannulation and exploration. This newer technique has seen isolated and limited use in the United States in pilot studies of two groups of women: women with nipple discharge and high risk women without discharge.¹ The current study was designed to examine the efficacy of fiberoptic ductoscopy (FDS) and ductal lavage in a larger series of 259 women, all with nipple discharge, to determine whether fiberoptic ductoscopy might offer a safe alternative to ductography in precisely diagnosing intraductal papillary lesions and guiding subsequent breast surgery.

METHODS

Patients

Informed patient consent and certification from the Institutional Human Subject Protection Committee of the Cancer Hospital, Shanghai Medical University, was obtained prior to all studies. Between October 1997 and December 1998, 259 female patients with nipple discharge gave their consent and were enrolled in the study. These patients received fiberoptic ductoscopy, ductal lavage, and subsequent breast surgery when indicated.

Fiberoptic Ductoscopy System (FDS)

FDS (FVS-3000, Fujikura Co., Ltd., Tokyo, Japan) consists of a silicafiberscope, a light source, an image monitor, and an image recorder, which can video record or directly photograph the observed image. An outer air channel of the fiberscope permits the installation and irrigation of saline washings and retrieval of cells from the ductal system of the breast. The outer diameter of the silicafiberscope we used was 0.72 mm and its maximum exploratory length was 6.5 cm. In select patients, we also used a double barrel lumen catheter designed to maximize ductal lavage.

FDS Procedure

The nipple and areola of the breast were cleaned with 70% ethanol and Povidone-iodine (Betadine) disinfectant. Bowman's lacrimal dilators with outer diameters of 0.35 mm and 0.45 mm, respectively, were lubricated with xylocaine jelly and then inserted into the

discharging nipple orifice to dilate the ostium of the lactiferous duct. The fiberscope then was inserted into the duct orifice. About 10 ml of normal saline then was perfused into the duct through the air channel of the fiberscope to insure the patency of the duct during the procedure. The lactiferous duct, lactiferous sinus, and the segmental duct and its branches were observed in orderly succession. The presence and appearances of any papillary lesions were noted, and the transductal distances from the nipple orifice to the proximal and distal borders of the lesions were measured and recorded.

Ductal Lavage and Cytology

The fiberscope was retracted and the instilled saline was retrieved and processed for cytology. A double lumen ductoscope was inserted in selected patients where the retrieved instilled saline was limited, and ductal washings were obtained by irrigation and processed for cytology. Cytological analysis consisted of standard cytospin preparations and Papanicolaou and Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL) staining. The cytological findings could be grouped into three categories: clumps of ductal cells (> 50 cells), clumps with atypia, and single ductal cells or small clumps.

Pathological Analysis

Patients then underwent breast surgery. Detailed histopathologic analysis of the extirpated tissues was carried out to evaluate the intraductal abnormalities present.

Statistical Analysis

Standard tests of significance were carried out. These included comparisons of differences among variables with the Student's *t* test for paired data and the Spearman rank based correlation to assess the relation between the variables. The log rank test was used to assess the univariate effect of certain variables on the presence or absence of an intraductal lesion. The Cox proportional hazards model was used to assess these effects after adjustment for other covariates.

RESULTS

FDS Screenings

Of the 259 patients studied, 92 (36%) were observed to have an intraductal papillary lesion (solitary, multiple, or diffuse) by FDS examination. In 167 (64%) patients, the FDS screening was negative. All of the 92 FDS positive patients had ductal lavage at the time of ductoscopy with cytological analysis and detailed histopathological examination of their subsequently re-

TABLE 1
Value of FDS Screening in Women with Nipple Discharge

Ductoscopy results	+Histopathology	-Histopathology
	Number of patients	Number of patients
+FDS (n = 92)	76	16
-FDS (n = 65)	12	53

Specificity 77%; Sensitivity 88%; Positive Predictive Value 83%; Negative Predictive Value 82%.

sected breast tissue specimen. All of the 167 FDS negative patients had ductal lavage, but only 65 elected to have surgery. Therefore, detailed histopathologic follow-up was available in only a subset of these FDS negative patients. Of the 92 patients with a positive FDS screening, 76 (83%) patients had intraductal papillary alterations (either solitary papilloma, multiple papilloma, or diffuse papillomatosis) on histopathological analysis, but 16 (17%) patients contained no such papillary alterations. Of the 65 patients with a negative FDS examination, only 12 (18%) had papillary alterations on histopathologic examination of the excised tissues. In half of these latter cases, the papillary alterations were small intraductal papillomas involving small to medium sized ducts probably beyond the fourth branch point of the ductal system, and, in the other half, there was diffuse papillomatosis of small ducts. For nipple discharge diagnosis, FDS screening exhibited high sensitivity and high specificity in detecting intraductal papillary lesions as well as high positive and high negative predictive values (Table 1).

The vast majority of women (> 95%) with nipple discharge have open ducts, so insertion of the ductoscope caused them no discomfort or pain. Therefore no anaesthesia was necessary. For the < 5% of women who were apprehensive, we applied a xylocaine jelly solution to the areola and nipple also. These patients also reported no discomfort or pain, and their initial apprehension faded during the procedure. The FDS technique should not be considered invasive. The flexible ductoscope entered patients' breasts but did not transgress the myoepithelial layer of the ductal system. We observed no untoward effects or complications in any of the 259 patients examined by FDS screening. Specifically, patients reported no persistent pain or discomfort from the procedure; we observed no mastitis, subareolar inflammation, or bleeding as a result of the procedure; and we saw no increase in nipple discharge following the procedure. Therefore, there was a high degree of patient

TABLE 2
Clinical Characteristics of Patients with Positive vs. Negative FDS Screening

Clinical characteristics	+FDS		-FDS	
	No. patients	Percentage	No. patients	Percentage
Menopausal status	92	100.0	167	100.0
Pre	68	73.9	125	74.8
Post	19	20.7	31	18.6
Castration	5	5.4	11	6.6
History (total)	92	100.0	167	100.0
Papilloma	4	4.4	5	3.0
Breast cancer	2	2.2	7	4.2
Negative	86	93.4	155	92.8
Localization-discharge	92	100.0	167	100.0
Unilateral	90	97.8	163	97.6
Bilateral	2	2.2	4	2.4
No. of discharge ducts	92	100.0	167	100.0
Single	88	95.7	160	95.8
Multiple	4	4.3	7	4.2
Duration of discharge	92	100.0	167	100.0
1 month	7	7.6	18	10.8
2-3 months	42	45.7	74	44.3
4-12 months	21	22.8	36	21.6
> 12 months	22	23.9	39	23.3
Discharge characteristic	92	100.0	167	100.0
Bloody ^a	30	32.6	31	18.6
Serosanguineous	16	17.4	38	22.7
Serous	42	45.6	87	52.1
Milky	2	2.2	6	3.6
Watery	2	2.2	5	3.0
Association with mass	92	100.0	167	100.0
No	80	87.0	140	83.8
Yes	12	13.0	27	16.2

^a Significant by univariate analysis, $P = 0.01$; insignificant in multivariate analysis, $P = 0.27$, when compared to FDS with respect to histopathology.

acceptance and compliance with the technique and an absence of possible complications.

Clinical Characteristics of the Patients in the Study

The mean age of all patients was 46 years (range 20-75 years). Before FDS screening, the average duration of nipple discharge had been 20 months and the longest duration had been 20 years. The clinical characteristics of the patients are shown in Table 2. Except for the presence of bloody discharge which was seen more frequently in the positive FDS group ($P = 0.01$ by univariate analysis), there were no statistically significant differences in any of the clinical parameters which distinguished the positive from the negative FDS group. In multivariate analysis, bloody discharge compared with FDS screening was less significant ($P = 0.027$) as a predictive marker for the presence of a histopathologically confirmed intraductal papillary lesion.

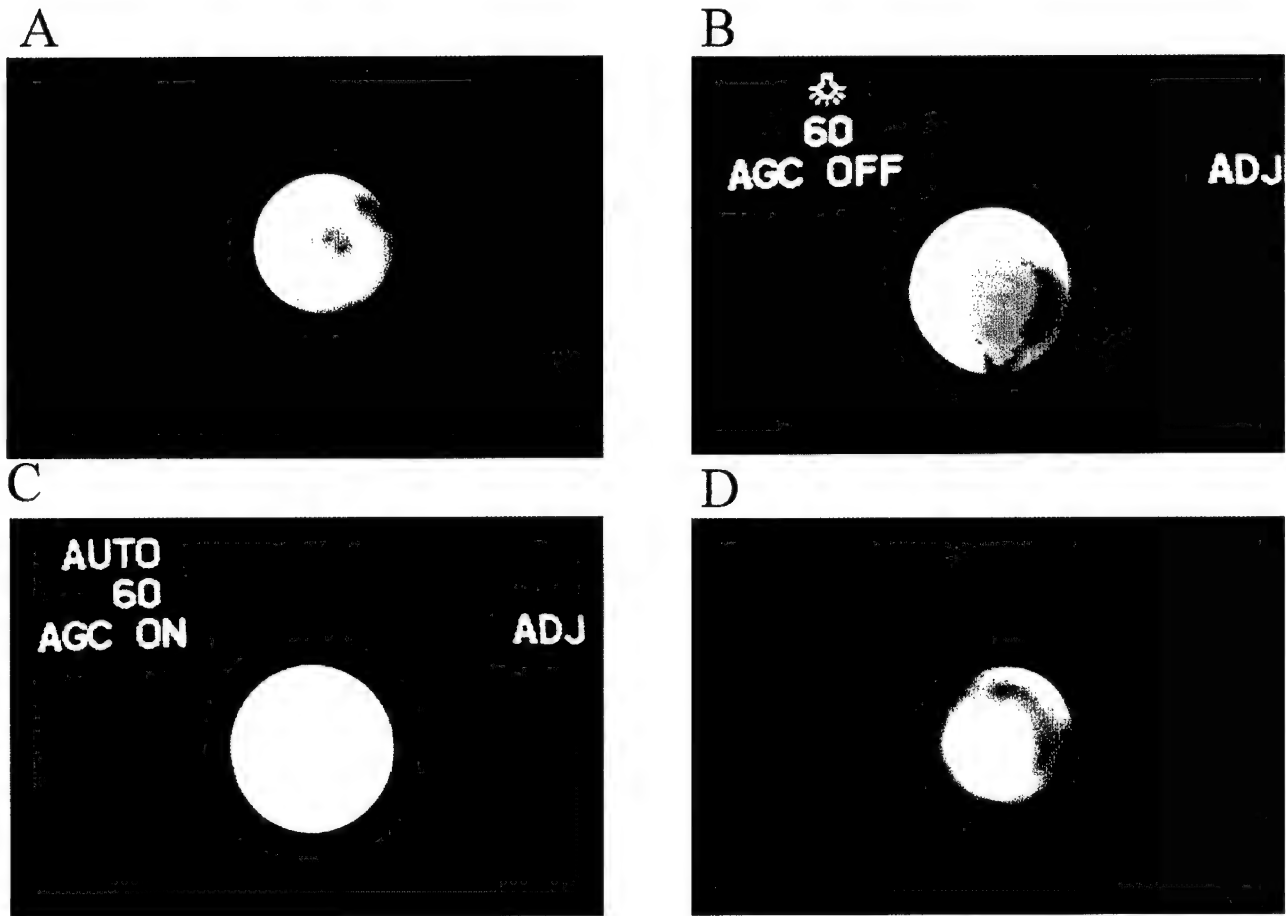


FIGURE 1. (A) Appearance of a normal segmental duct at the first bifurcation where two lumens are present; (B) Immediately after the first bifurcation of the same case, a large reddish intraductal proliferation representing a solitary intraductal papilloma was observed; (C) In a different case, a large intraductal proliferation was present as a rounded white mass; (D) In yet another case the intraductal papilloma was present as an obstructing intraluminal mass with a thin rim of patency still present at 12–4 o'clock. The proximal and distal margins of the papilloma could be measured in terms of transductal distances from the nipple orifice and used to guide subsequent surgery.

The Ductoscopy Technique, Instrumentation, and Analysis

The learning curve for cannulation of the ducts, advancing the ductoscope, and observing the lesion was fairly steep with incremental gains in skill seen during the first 10 cases. After that point, the examiner reached a steady state of maximum versatility with the technique. It should be pointed out that the specialists who performed the technique were surgeons with basic skills in the use of instrumentation of this sort. The cost of the equipment was \$400,000 (U.S. dollars) for the silicafiberscope, the light source, the image monitor, and the image recorder. Each fiberscope could be reused for 100 patients. The cost of a fiberscope replacement was \$1000 (U.S. dollars).

Experienced pathologists and cytologists could learn easily how to interpret cells obtained by ductal lavage, but as with any new technique, they first must

learn the different cytological features that were present in cells obtained by ductal lavage and apply these newly learned criteria. The cells obtained were somewhat different in morphology and far greater in number than cells present in routine nipple aspirates.

Observation of the Breast Ducts with FDS

Normal ductal cavities presented appearances ranging from lustrous pale yellow to pink and were observed to exhibit ring folds on the duct walls. The main segmental duct could readily be observed to bifurcate into primary branches (Figure 1A). The appearance of an intraductal papillary lesion under FDS examination was either red, yellow, or ash-gray. The lesion appeared as a polypoid mass projecting into the lumen of the duct (Figures 1B, 1C, 1D). The majority of intraductal papillary lesions were present either as sol-

2A

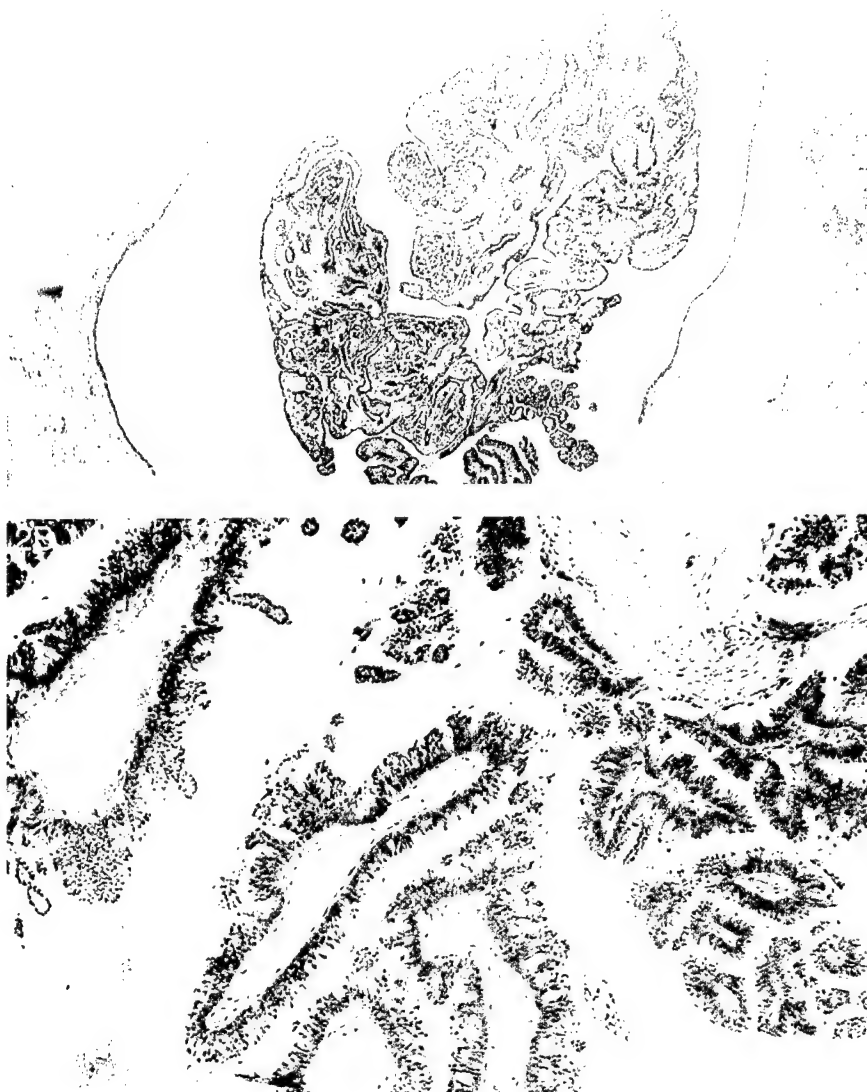


FIGURE 2. (A) Appearance of the classic solitary intraductal papilloma depicted in Figure 1B is demonstrated by histopathology of the resected specimen; (B) four (4.5%) of the intraductal papillary lesions in the study exhibited areas of atypia or areas of frank carcinoma; the lesion depicted represented an atypical intraductal papillary proliferation that was part of an extensive intraductal papillary carcinoma from a case where there also was adjacent invasive carcinoma. (C) This atypical papillary proliferation gave rise to abnormal cytology in ductal lavage.

itary lesions (Figure 2A) or multiple lesions in a single ductal system (Table 3), but a very small minority occurred in two different ductal systems, either unilaterally or bilaterally (Table 2). In 3% of the positive FDS patients, no discrete intraductal papillary lesion was observed, but rather a diffuse ductal thickening was noted. The presence of this diffuse intraductal thickening corresponded to the finding of diffuse papillomatosis on histopathologic study.

The shortest transductal distance from intraductal papillary lesion to the nipple orifice was 0.5 cm, the longest was 6 cm, and the average was 2.7 cm. The transductal distances to the proximal and distal margins of the intraductal lesions were recorded and used along with intraductal catheters at the time of surgical excision. The intraductal lesions occurred in the main segmental duct and the first, second, third, and fourth branches in decreasing frequency (Table 4).

2C



FIGURE 2. (continued)

TABLE 3
Numeric Distribution of Lesions in Patients with Positive FDS

Number of lesions	Number of patients	Percentage (%)
One	68	74.0
Two	15	16.3
Three	6	6.5
Diffuse	3	3.2
Total	92	100.0

TABLE 4
Geographic Distribution of Lesions in Patients with Positive FDS

Location	Number of lesions	Percentage (%)
Segmental	34	29.8
First branch	50	43.9
Second branch	20	17.5
Third branch	9	7.9
Fourth branch	1	0.9
Total	114	100.0

Cytological Analysis of Ductal Lavage

Cytological findings were grouped into three categories: clumps of ductal cells (> 50 cells), clumps with atypia (based on nuclear pleomorphism, chromatin staining, and size), and single ductal cells or small clumps. For the purposes of this study, we assumed that large ductal clumps reflected the exfoliation of an intraductal papillary lesion and that single ductal cells reflected the absence of the same. The positive and negative predictive values of cytologic analysis alone

TABLE 5
Value of Cytology in Positive vs. Negative FDS Screening

Cytology	Overall total (n = 157) no. of patients		+FDS (n = 92) no. of patients		-FDS (n = 65) no. of patients	
	+Path	-Path	+Path	-Path	+Path	-Path
Clumps > 50 cells	35	15	32	6	3	9
Atypical clumps	4	0	4	0	0	0
Single cells	52	51	45	5	7	46
Specificity		77%		45%		84%
Sensitivity		43%		44%		30%
Positive Predictive Value		72%		86% ^a		25%
Negative Predictive Value		50%		10%		87% ^a

^a Value greater than FDS alone

for the presence/absence of an intraductal papillary lesion were 72% and 50%, respectively (Table 5). These cytologic values were less than the corresponding predictive values of FDS which were 83% and 82%, respectively (Table 1). However, when cytologic analysis was combined with FDS, the positive predictive value of the combination was 86% and the negative predictive value was 87% (Table 5). Therefore cytologic analysis combined with FDS screening provides the best preoperative assessment of the likelihood that an intraductal papillary lesion is the cause of nipple discharge.

All four cases in the positive FDS group that exhibited atypical clumps of ductal cells were associated with intraductal papillary lesions showing atypical features: either areas of atypical ductal hyperplasia (two cases) or ductal carcinoma in situ (one case), or frankly intraductal papillary carcinoma (one case) (Figure 2B). Therefore the presence of atypical clumps (Figure 2C) together with a positive FDS screening exhibited a 100% positive predictive value for an atypical intraductal papillary lesion.

DISCUSSION

This study demonstrates the value of FDS and ductal lavage cytology as a screening and preoperative procedure in women with nipple discharge. Certainly clinical characteristics do not distinguish the patients whose nipple discharge is due to intraductal papillary lesions from those due to other causes. Furthermore, clinical characteristics do not distinguish the type of papillary lesion, its singularity, multiplicity, or its degree of atypism. FDS and ductal lavage cytology do.

FDS is gaining in popularity as thinner, longer, and more flexible fiberoptic scopes become available to permit better observation of the ductal system of the breast. Breast ductoscopy has evolved slowly over the past decade. In 1988, through a rigid endoscope of 1.7 mm in its outer diameter, Teboul⁴ was first to succeed in observing the duct cavity under the guidance of ultrasound. In 1989, Makita⁵ established blind intraductal biopsy of the breast for pathologic diagnosis of nipple discharge through a rigid endoscope of 1.25 mm in its outer diameter. The endoscope that he used was similar to an arthroendoscope. Later Okasaki,⁶ in cooperation with the Fujikura Company, developed a breast fiberoptic ductoscopy system making it possible to observe directly the duct cavities and duct walls and to project them onto a screen. This technique allowed for observation of even minute intraductal lesions.

In patients with nipple discharge, many are found not to have an intraductal papillary lesion. In most studies, intraductal papilloma and papillomatosis account for, at most, 35%–48% of cases of nipple discharge. By papillomatosis, we mean a diffuse multicentric hyperplasia involving major ducts. We use the term papillomatosis because many of the lesions were papillary in appearance, but we acknowledge that this papillomatosis is a form of epithelial hyperplasia. In our study, only 92 of 259 (36%) patients with nipple discharge had demonstrable intraductal papillary lesions by FDS screening. Breast duct endoscopy, therefore, can screen these patients effectively. If it finds no demonstrable lesion, patients may be spared surgery or may choose single duct ligation rather than exci-

sion. Conversely, positive FDS would suggest surgery and would dictate the type and extent of surgery. A variety of clinicopathologic subtypes of papillary lesions, which include solitary and multiple papillomas, diffuse papillomatosis, papillomas with atypical ductal hyperplasia or focal DCIS, and papillary carcinomas have been observed in the ductal system.^{7,8,9} All of these subtypes were observed by FDS in our study and sampled by ductal lavage. FDS showing multiple papillomas or diffuse papillomatosis suggests a more extensive duct excision than FDS showing a single intraductal papilloma. FDS with atypical cytology suggests a more extensive resection of subareolar tissues since the presence of atypical ductal hyperplasia, focal DCIS or frank intraductal papillary carcinoma merits wider excision to achieve completely negative surgical margins.

The ductal system of the breast illustrates a significant degree of branching morphogenesis. Intraductal papillary lesions can exist in any branch. Since the direct observation of intraductal lesions is limited by two ductoscopy parameters, its length of 6.5 cm and its outer diameter of 0.72 mm (which limits access to ducts smaller than this diameter), it is conceivable that peripheral intraductal lesions causing nipple discharge are beyond the reach of the ductoscope and could be missed. In fact, for the 12 patients in our study who had a negative FDS screening but papillary lesions on histopathologic examination, their histopathologic analysis revealed either small papillomas in small to medium sized ducts, ducts probably beyond the fourth branch or diffuse papillomatosis in small ducts. This peripheral distribution of some intraductal papillary lesions probably explains the FDS examination false negative rate of 18%. As longer, thinner, and more flexible probes become available, this false negative rate likely will decrease.

Intraductal papillary lesions are usually minute lesions growing in the ductal wall. They are fragile and can easily exfoliate. This may explain the difficulty in finding papillary lesions in surgical specimens on gross examination and the absence of suspected papillary lesions on histopathologic examinations of resected specimens. This fragility and minuteness might explain, in part, why 17% of patients with positive FDS had no demonstrable lesion on histopathologic examination. Conceivably, in some of these patients ductoscopy itself could have exfoliated the lesion so that no lesion remained when histological analysis was carried out. Therefore, the 17% false positive rate may be considerably lower.

Although this study did not compare ductoscopy with ductography specifically, it seems intuitive that ductoscopy provides advantages over ductography in

TABLE 6
Historical Comparisons of Breast Ductoscopy with Breast Ductography

Feature	Ductoscopy	Ductography
Proximal limits of lesion	Yes	Yes
Distal limits of lesion	Yes	No
Duct obstruction required	No	Yes
Direct observation	Yes	No
Precise distances	Yes	No
Cytologic analysis	Yes	No

the routine management and examination of patients with nipple discharge. Ductoscopy allows direct observation of lesions and not just indirect shadowing. Ductoscopy, unlike ductography, allows more precise localization and mapping of the lesion within the duct. This mapping is not dependent on ductal obstruction and includes both proximal and distal margins of the lesion and not just the former. Ductoscopy, unlike ductography, allows intraductal sampling of the ductal cells for cytologic analysis which in turn increases both positive and negative predictive values. The historical advantages of ductoscopy over ductography are summarized in Table 6. In terms of specificity, sensitivity, positive and negative predictive values in detecting an intraductal lesion in patients with nipple discharge, we are currently conducting a prospective randomized trial comparing ductoscopy with ductography. However before conducting and reporting on such a comparative study, we felt it important to report first the positive results with ductoscopy itself.

The unique advantages of ductoscopy should result in its ability to direct and limit subsequent breast surgery more precisely, so that surgery can remove the offending lesion and yet spare as much uninvolved breast tissue as possible.

Breast ductoscopy is very inexpensive by present

standards. In mainland China, it costs only \$50 U.S. dollars. In the United States, it probably would cost approximately \$200. Certainly this screening technique is no more expensive than ductography and only slightly more expensive than nipple smear aspiration cytology.

Ductoscopy in the future may permit direct intraductal ablation of the lesion by either mechanical or chemical means which would spare surgery altogether in selected patients. The benefit of intraductal ablation certainly would be cost effective when compared to surgery.

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Fiberoptic ductoscopy for breast cancer patients with nipple discharge

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Abstract

Background: Breast cancer and precancer are thought to originate in the lining of the milk duct, but until recently, we have not had direct access to this area other than in tissue removed blindly by core biopsy or fine-needle aspiration. Fiberoptic ductoscopy (FDS) is an emerging technique that allows direct visual access to the ductal system of the breast through nipple orifice cannulation and exploration. To date, this technique has been used only in pilot studies. Previously, we have demonstrated that fiberoptic ductoscopy in patients with and without nipple discharge is a safe and effective means of visualizing the intraductal lesion. When combined with cytology, it is a screening technique that has high predictive value. **Methods:** We applied ductoscopy to 415 women with nipple discharge with the specific intent of detecting those patients with nipple discharge who had intraductal carcinoma (DCIS) as the basis of their discharge. **Results:** In this cohort of patients, ductoscopy was successful in visualizing an intraductal lesion in 166 patients (40%). In these cases, ductal lavage following ductoscopy increased the yield of cytologically interpretable ductal epithelial cells 100-fold compared to discharge fluid alone. In the majority of these patients, FDS examination detected lesions that had the appearance of typical papillomas. However, in 10 patients, the intraductal lesion exhibited one of several atypical features, including bleeding, circumferential obstruction, and gross fungating projections. In eight of these patients, the subsequent histopathology turned out to be DCIS. In two of these eight patients, endoscopic biopsy revealed cytologically malignant cells; in two others, ductal lavage (washings) revealed cytologically malignant cells. In three additional patients, although FDS examination uncovered a typical papilloma that was not biopsied, ductal lavage (washings) revealed cytologically malignant cells. On surgical pathology review of the extirpated lesions, all 11 patients were subsequently shown to have DCIS. Of these 11 cases of DCIS that were initially detected with a combination of FDS and ductal lavage cytology, six were completely negative on mammogram and physical exam. **Conclusion:** Although nipple discharge is an unusual presentation for DCIS, in patients with nipple discharge, FDS with ductal lavage cytology is a useful technique for diagnosing DCIS prior to definitive surgery.

Keywords: Nipple discharge, Breast cancer, Fiberoptic ductoscopy, Intraductal carcinoma, Cancer

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Breast cancer and precancer are thought to originate in the lining of the milk duct or lobule. Yet until recently we have not had direct access to this area other than by examining tissue removed at biopsy. Fiberoptic ductoscopy (FDS) is an emerging technique that allows direct visual access to the ductal system of the breast through nipple orifice cannulation and exploration [3, 7]. In previous studies [3, 8], we have used FDS successfully in women with and without nipple discharge.

Although nipple discharge is relatively common and usually benign in origin, it can in fact herald the onset of intraductal carcinoma of the breast (DCIS). The incidence of DCIS in patients with nipple discharge varies from 1% to 10% [5]. The diagnostic workup of patients with nipple discharge usually includes clinical history, physical examination, mammography, ductography, and nipple discharge cytology but not FDS [2]. In a previous study, we demonstrated that in patients with nipple discharge, FDS is a safe and effective means of visualizing the intraductal lesion. Moreover, when combined with ductal lavage cytology, it is a screening technique that has a high predictive value [8].

In the present study, we applied ductoscopy to 415 women with nipple discharge with the specific intent of detecting those patients whose nipple discharge was the result of DCIS.

Materials and methods

Patients

Informed patient consent and certification from the Institutional Human Subject Protection Committee of the Cancer Hospital, Shanghai Medical University, was obtained prior to all studies. Between October 1997 and March 2000, 415 patients with nipple discharge consented and were enrolled in the study. These patients were subjected to FDS, endoscopic biopsy when indicated, ductal lavage with cytological analysis, and subsequent surgery when appropriate.

Fiberoptic ductoscopy system (FDS)

The FDS setup (FVS-3000; <Author Query 1: Fujikura> Co. Ltd.) consisted of a silica fiberscope, a light source, an image monitor, and an image recorder, which was capable of videorecording or directly photographing the observed image. An outer air channel of the fiberscope allowed for the installation and irrigation of saline washings and the retrieval of cells from the ductal system of the breast. The outer diameter of the silica fiberscope was 0.72 mm; its maximal exploratory length was 6.5 cm. In select patients, a double-barrel lumen catheter designed to maximize ductal lavage was also used.

FDS procedure

The nipple and areola of the breast were cleaned with 70% ethanol and povidone-iodine disinfectant (Betadine). Bowmann's lacrimal dilators with outer diameters of 0.35 mm and 0.45 mm, respectively, were inserted sequentially into the discharging nipple orifice to dilate the ostium of the lactiferous duct. The fiberscope was then inserted into the duct orifice.

The procedure is not at all uncomfortable for the patients. When the patients have nipple discharge, their nipple orifice is already somewhat dilated and easily accessible. The majority of patients required

no anesthesia at all; a minority required only the local lubrication of the nipple orifices with xylocaine jelly. No other forms of local or general anesthesia were used. The patients were not given IV sedation. This is a painless procedure.

About 10 ml of normal saline was then perfused into the duct through the air channel of the fiberscope to ensure the patency of the duct during the procedure. The lactiferous duct, lactiferous sinus, and the segmental duct and its branches were visualized in succession. The presence and appearance of any papillary lesions were noted, and the transductal distances from the nipple orifice to both the proximal and distal borders of the lesions were measured and recorded. The presence of atypical papillary lesions were specifically identified by the presence of bleeding, circumferential obstruction, or gross fungating projections.

Endoscopic biopsy

Endoscopic biopsy was performed when the presence of atypical papillary lesions was noted and when the biopsy was technically feasible. The fiberscope, which was covered with an outer cylinder, was inserted into the location of the suspected lesion through the ostium of the duct. Under the guidance of FDS, the outer cylinder was brought just up to the lesion. After fiberscope was removed, a very thin inserted syringe was used to aspirate the lesion through the outer cylinder. With this technique, the fine-needle aspiration produced tissue fragments having the appearance of endoscopic biopsy specimens. These specimens were placed in 10% formalin for cytopathological examination.

Ductal lavage and cytology

The fiberscope was retracted, and the instilled saline was retrieved and processed for cytology. A double-lumen ductoscope was inserted in selected cases where retrieval of the instilled saline was limited, and ductal washings were obtained by irrigation and processed for cytology. Cytological analysis consisted of standard cytospin preparations and Pap and Diff-Quick staining. The cytological findings were grouped into the four following categories: clumps of ductal cells (>50 cells), clumps with mild atypia, clumps with severe atypia (cytologically malignant cells), and single ductal cells or small clumps. The number of ductal epithelial cells obtained on ductal lavage were compared to the number spontaneously present in the nipple discharge. For the purposes of this study, the four cytological categories were reduced to two—cytologically malignant and cytologically benign.

Pathological analysis

Select patients were subsequently subjected to breast surgery. Detailed histopathological analysis of the extirpated tissues was carried out to evaluate the intraductal abnormalities present.

Statistical analysis

Standard tests of significance were carried out. These included comparisons of differences among variables with the two-tailed Student's *t*-test and the Spearman's rank-based correlation to assess the relationship between the variables. The log-rank test was used to assess the univariate effect of certain variables on the presence or absence of an atypical vs typical intraductal lesion. Cox's proportional hazards model was used to assess these effects after adjustment for other covariates.

Results

Overview of findings

In this cohort of patients, ductoscopy was successful in visualizing an intraductal lesion in 166 patients (40%). In the majority of these patients, FDS examination detected lesions having the appearance of typical papillomas. However, in 10 patients, the intraductal lesion exhibited one of several atypical features, including bleeding, circumferential obstruction, and gross fungating projections (Fig. 1). In eight of these patients, the subsequent histopathology turned out to be DCIS (Table 1). In four of these eight patients, cytologically malignant cells were present either on endoscopic biopsy or ductal lavage (Fig. 2). In three additional patients, although FDS examination revealed a typical papilloma that was not biopsied, ductal lavage (washings) revealed cytologically malignant cells. All 11 patients were subsequently shown to have DCIS on surgical pathology review of the extirpated lesions.

Clinical characteristics of the breast cancer patients with nipple discharge

Eleven of 415 patients with nipple discharge (2.7%) were therefore found to have DCIS. The mean age of all 11 DCIS patients was 43 years old (range, 27–56). The average duration of nipple discharge was 3 months and the longest duration was 8 months. The clinical characteristics of the patients are summarized in Table 2: The most prominent symptom of the patients in this study was spontaneous unilateral bloody nipple discharge from a single duct.

FDS findings

The intraductal lesions observed in the cases of DCIS tended to lie more distally than their benign papilloma counterparts observed both in this study and in a previous study [8]. The geographic distribution of the eight DCIS lesions detected by FDS were mainly in the first and second branches of the ductal system (Table 3) as opposed to the segmental and first branch, as was the case with intraductal papillomas. Predictably, the transductal distances from the nipple orifice to the lesion were greater for DCIS than for intraductal papillomas. The shortest transductal distance from the suspected lesion to the nipple orifice was 1.5 cm, the longest was 5 cm, and the average was 3.3 cm. In contrast, for intraductal papillomas, the shortest distance was 0.5 cm and the average was 2.7 cm.

The appearance of the eight DCIS lesions was quite different from the typical intraductal papilloma. The DCIS lesions exhibited bleeding (Fig. 1a), circumferential ductal obstruction (Fig. 1b), and irregular fungating masses projecting from the lumen (Fig. 1c). In contrast, the appearance of the intraductal papilloma was polyplike, with smooth borders and a narrow stalk (Fig. 1d).

Endoscopic biopsy findings

In two of the eight patients whose FDS examination revealed an atypical intraductal lesion, we were able to obtain a satisfactory endoscopic biopsy, which revealed tissue fragments of cytologically malignant cells (Fig. 2).

Ductal lavage cytology findings

Ductal lavage following ductoscopy in all cases that were studied dramatically increased the yield of cytologically interpretable ductal epithelial cells; it was 100-fold greater than the yield obtained with discharge fluid alone. The number of cells obtained averaged 5000 cells per washed duct, as compared to 50 in discharge fluid alone. The positive predictive value of FDS alone in detecting DCIS was 80%

(Table 1), but when combined with ductal lavage cytology, the positive predictive value increased to 100% (Table 4).

Comparison of FDS and ductal lavage with physical examination and mammography

Of the 11 cases of DCIS that were initially detected with a combination of FDS and ductal lavage cytology, six cases were completely negative on mammogram and physical exam (Table 2).

Discussion

FDS is gaining in popularity as thinner, longer, and more flexible fiberoptic scopes become available to permit greater visualization of the ductal system of the breast. Breast ductoscopy has evolved slowly over the past decade. In 1988, using a rigid endoscope with a 1.7-mm outer diameter [9], Teboul was the first investigator to succeed in observing the duct cavity under the guidance of ultrasound. In <Author Query 3: 1989>, Makita et al. [4] established the use of blind intraductal biopsy of the breast for the pathologic diagnosis of nipple discharge through a rigid endoscope with a 1.25-mm outer diameter. The endoscope that they used was similar to an arthroendoscope. Later, Okazaki et al. [6], in cooperation with the Fujikura Company, developed the breast fiberoptic ductoscopy system. This system made it possible to visualize the duct cavities and duct walls directly and project them onto a screen. This technique allowed for the visualization of even minute intraductal lesions.

Using this technique [8], we were able to effectively screen women with nipple discharge. In our initial study, we applied ductoscopy to 259 women with nipple discharge and analyzed the visual findings, the cytological washings, and the subsequent histopathology. In 92 of these women (36%), fiberoptic ductoscopy was successful in visualizing an intraductal papillary lesion. Of these visualized cases, 68 (74%) had a single papilloma, 21 (23%) had multiple discrete papillomas, and three (3%) had diffuse intraductal thickening that corresponded to diffuse papillomatosis on histopathological analysis. The overall positive predictive value of FDS screening was 83%. Ductal washings done at the time of ductoscopy were effective at obtaining representative exfoliated ductal cells that could be evaluated for the presence of clumps (>50 cells) or single ductal cells. The presence of clumps with positive FDS increased the positive predictive value to 86%. We concluded that fiberoptic ductoscopy offered a safe alternative to ductography in guiding subsequent breast surgery in the treatment of nipple discharge.

Because nipple discharge can occasionally herald the onset of intraductal carcinoma of the breast (DCIS), we decided to determine whether FDS and postductoscopy lavage might prove useful in determining which patients <Author Query 4: had> nipple discharge that was symptomatic of DCIS. With this approach, 11 of 415 cases were shown to have DCIS prior to surgical extirpation of the lesion.

Bauer et al. reported that when DCIS is associated with nipple discharge, nipple discharge becomes a clinical marker for locally extensive disease [1]. When the discharge is due to DCIS, extensive intraductal spread of the disease may preclude breast conservation. Our results concerning the locations of the lesions and their extent of ductal involvement were not significantly different from what we previously observed for intraductal papillomas [8]. Although DCIS is located slightly more distally from the nipple orifice, it does not involve more than one ductal system and its extent of ductal involvement can be defined with FDS. With the help of FDS, we can determine the location and extent of the DCIS before surgery; this information can be of tremendous assistance in planning breast conservation surgery.

Some investigators have implied that ductography is the best method for preoperative evaluation of the nature and site of the lesion causing nipple discharge, but the accuracy rates reported for the detection of DCIS in the setting of nipple discharge were only 39% and 70%, respectively [4]. The main reason for the low rates was that ductography could not discriminate between DCIS and papilloma. In our study, we show that FDS can discriminate effectively between DCIS and intraductal papilloma. Although we did not specifically compare ductoscopy with ductography in the management of patients with nipple discharge, it seems obvious that ductoscopy provides advantages over ductography in the routine management and workup of patients with nipple discharge (Table 5).

Ductoscopy allows direct visualization of the lesion <Author Query 5: rather than yielding just an> indirect shadow image. In comparison to ductography, ductoscopy allows for more precise localization and mapping of the lesion within the duct. This mapping is not dependent on the presence of obstruction. Ductoscopy, unlike ductography, permits intraductal sampling of the ductal cells through endoscopic biopsy and increases exfoliation of the cells in ductal lavage following ductoscopy 100-fold over that which occurs spontaneously in nipple discharge. In contrast, ductography obscures cytological detail and renders cytological evaluation impossible.

One of our important observations was that >50% of the patients with nipple discharge who had a DCIS lesion visible on FDS and/or detectable by ductal lavage had a negative physical examination and a negative mammogram (Table 2). In a recent study of women presenting with nipple discharge [1], mammography revealed subareolar microcalcifications in 28% and tissue density in 14%, but it was completely negative in 52% of cases. It is especially in these latter cases that alternative detection methods such as FDS and ductal lavage would find utility. This study dealt with women who present with nipple discharge and the DCIS they harbor. But FDS and ductal lavage may hold greater promise as screening techniques for all women at high risk for breast cancer who are persistently negative on mammogram and physical exam.

In our previous study [8], we demonstrated that in 36% of women with nipple discharge, fiberoptic ductoscopy was successful in detecting an intraductal papillary lesion, with an overall positive predictive value of 83%. Therefore, from the standpoint of numbers of patients, the technique is cost-effective. Most of these intraductal lesions were intraductal papillomas. Only a minority were intraductal carcinomas. But we are advocating the technique for all women with nipple discharge, not just those whose discharge is due to intraductal carcinomas.

The fiberoptic ductoscope and related equipment cost US \$40,000. This amount represents a one-time cost for the silica fiberscope, the light source, the image monitor, and the image recorder. Each fiberscope can be reused for 100 patients. The cost of a fiberscope replacement is US \$1000. Considering that patients with nipple discharge whose ductoscopy examination visualized a lesion were spared the cost of ductography and that patients whose ductoscopy/lavage were negative were spared the cost of surgical excision, the technique is quite cost-effective.

<Author Query 6: We cannot yet address the utility of> diagnosing these lesions at a slightly later time if ductoscopy/lavage is not performed but nipple discharge is followed by more frequent screening mammograms or ultrasound examinations. However, considering that DCIS becomes a breast-threatening disease as it grows, and considering that the risk of invasion increases with size and duration, it seems intuitive that earlier diagnosis of DCIS is desirable.

The two techniques of ductoscopy and ductal lavage are intimately associated and should not be separated. Both ductoscopy and ductal lavage are done easily, and ductoscopy facilitates ductal lavage because it dilates the nipple orifices further. Ductoscopy both visualizes the lesion and determines its location and extent—determinations that cannot be made with ductal lavage alone. The cost of doing both techniques at the same time is no greater than doing either technique alone. Since we do not know in advance what the findings will be, it is not possible, nor would it be desirable, to stratify the patients into receiving only ductoscopy or ductal lavage.

Acknowledgments

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Tables

Table 1. + (suspicious) vs – (nonsuspicious) fiberoptic ductoscopy (FDS) ^{a,b} in the detection of intraductal carcinoma (DCIS) in women with nipple discharge

Table 2. Clinical characteristics of the patients with intraductal carcinoma (DCIS) identified by fiberoptic ductoscopy (FDS) and/or ductal lavage cytology

Table 3. Anatomical distribution of lesions in patients with intraductal carcinoma (DCIS) identified by fiberoptic ductoscopy (FDS)

Table 4. Value of cytology in + (suspicious) vs – (nonsuspicious) fiberoptic ductoscopy (FDS) examination

Table 5. Comparison of breast ductoscopy with breast ductography

Figures

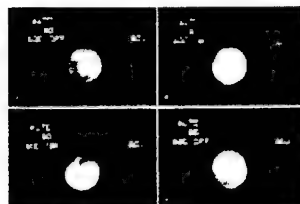


Figure 1

Figure 1 (large scale)

Fig. 1. Various appearances of the atypical intraductal papillary lesion suggesting intraductal carcinoma (DCIS): **a** a bleeding lesion, **b** a circumferential obstructive lesion with a roughened surface resembling a snow slide on a mountain, **c** an irregular fungating mass, **d** <Author Query 2: a typical intraductal papilloma, which had a narrow base and a smooth border and appeared to be a polyp>.



Figure 2

Figure 2 (large scale)

Fig. 2. An endoscopic biopsy specimen of intraductal carcinoma (DCIS) obtained through the ductoscope showing a clump of cytologically malignant cells.

Queries

Author Query 1

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Table 1. + (suspicious) vs – (nonsuspicious) fiberoptic ductoscopy (FDS)^{a,b} in the detection of intraductal carcinoma (DCIS) in women with nipple discharge

Ductoscopy results	+ histopathology (no. of patients)	–histopathology (no. of patients)
+ FDS (<i>n</i> = 10)	8	2
– FDS (<i>n</i> = 156)	3	153

^a + FDS examination refers to the finding of an atypical papillary lesion; – FDS examination refers to the finding of a typical papillary lesion. All patients had a positive FDS examination from the standpoint of an intraductal lesion.

^b Specificity, 99%; sensitivity, 73%; positive predictive value, 80%; negative predictive value, 98%

Table 2. Clinical characteristics of the patients with intraductal carcinoma (DCIS) identified by fiberoptic ductoscopy (FDS) and/or ductal lavage cytology

Clinical characteristics	No. of patients	Percentage (%)
Menopausal status	11	100
Premenopausal	6	54.5
Postmenopausal	5	45.5
History	11	100
Family history of nipple discharge	1	9.1
Family history of breast cancer	3	27.3
Negative history	7	63.6
Localization of discharge	11	100
Unilateral	11	100
Bilateral	0	0
No. of discharge ducts	11	100
Single	10	100
Multiple	0	0
Duration of discharge	11	100
≤ 1 mo	5	45.4
2–3 mo	3	27.3
4–8 mo	3	27.3
Discharge characteristic	11	100
Bloody ^a	6	54.6
Serosanguineous	4	36.3
Serous	1	9.1
Mass—physical examination	11	100
No	8	72.7
Yes	3	27.3
Mammography	11	100
Microcalcifications	2	18.2
Architectural distortion (mass)	3	27.3
Negative	6	54.5

^a Significant by univariate analysis, $p = .01$; insignificant in multivariate analysis, $p = .20$ when compared to FDS with respect to histopathology

Table 3. Anatomical distribution of lesions in patients with intraductal carcinoma (DCIS) identified by fiberoptic ductoscopy (FDS)

Location	No. of lesions	Percentage (%)
Segmental	1	12.5
First branch	3	37.5
Second branch	3	37.5
Third branch	1	12.5
Fourth branch	0	0
Total	8	100.0

Table 4. Value of cytology in + (suspicious) vs – (nonsuspicious) fiberoptic ductoscopy (FDS) examination^a

Cytology	Overall (n = 166)		+ FDS (n = 10)		– FDS (n = 156)	
	+ Path	– Path	+ Path	– Path	+ Path	– Path
Malignant cells (no. of patients)	7	0	4	0	3	0
Benign cells (no. of patients)	4	155	4	2	0	153
Specificity	100%	100%	100%			
Sensitivity	64%	50%	100%			
Positive predictive value	100%	100% ^b	100%			
Negative predictive value	97%	33%	100% ^b			

^a Path, pathology + FDS examination refers to the finding of an atypical papillary lesion; – FDS examination refers to the finding of a typical papillary lesion. All patients had a positive FDS examination from the standpoint of an intraductal lesion.

^b Value greater than FDS alone (see Table 1)

Table 5. Comparison of breast ductoscopy with breast ductography

Feature	Ductoscopy	Ductography
Proximal limits of lesion	Yes	Yes
Span of the lesion	Yes	No
Distal limits of lesion	Yes	No
Duct obstruction required	No	Yes
Direct visualization	Yes	No
Precise distances	Yes	No
Endoscopic biopsy	Yes	Yes
Cytologic analysis	Yes	No

chemotherapy. A phase II trial has been started to assess the therapeutic efficacy of this combination.

We thank Priscilla Gray, clinical research associate, for help in enrolment of the patients in the STEPS programme and for help in the follow-up of patients.

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Breast-cancer diagnosis with nipple fluid bFGF

Yeheng Liu, Jing Liang Wang, Helena Chang, Stanford H Barsky, Mai Nguyen

Early diagnosis of breast cancer is the key to extending survival of breast-cancer patients. We found that the concentrations of nipple fluid bFGF (basic fibroblast growth factor) was significantly increased in breast-cancer patients compared with concentrations in controls (1717 ng/L [SD 706] vs 19 ng/L [19]; Student's *t* test *p*=0.027). Measurement of bFGF in nipple fluid could be a useful diagnostic tool for breast cancer, and deserves further study. Breast cancer is the most frequently diagnosed cancer in American women. The key to extending survival is early diagnosis. Early detection through screening mammography saves lives; however, mammography fails to detect 20% of breast cancers. Breast biopsy samples from women with abnormal mammograms confirm cancer in only 10-20% of cases. Furthermore, current serum tumour markers are not useful in diagnosing breast cancer.¹

The process of angiogenesis has a critical role in breast tumour growth and metastasis. Studies from our laboratory and from other institutions have shown that angiogenic factors can be significantly increased in the serum and urine of breast-cancer patients.² The concentration of bFGF (basic fibroblast growth factor) has been shown to correlate with the disease stage of the tumour.³ However, it cannot be used as a screening tool because there is significant overlap between normal individuals and cancer patients. We assessed whether the concentration of these angiogenic peptides in the nipple fluid, which have half lives measured in min, can be used as a diagnostic tool for breast cancer. Because breast cancer arises from the epithelium of the terminal duct lobular unit, we expected that the fluid secreted into the breast ducts would contain a much higher concentration of these angiogenic factors than serum or urine.

We measured bFGF by ELISA (R&D Systems, Minneapolis, USA) in the nipple fluid from ten patients without benign breast conditions (controls), four lactating

women, and ten women with stage 1 or 2 breast cancer (table). We were able to obtain nipple fluid from these 24 women but attempted to get samples from 30 women (80% success rate). This study was approved by the UCLA Institutional Review Board, and all participants signed informed consent forms. The detection limit was 100 ng/L. Nipple fluid from controls had significantly lower concentrations of bFGF compared with nipple fluid from cancer patients (19 ng/L [SD 19] vs 1717 ng/L [706]; *p*=0.027 Student's *t* test). There was very little overlap in the values of bFGF measured in these two groups. The one cancer patient with undetectable concentrations of bFGF in the nipple fluid had already had surgical resection of the cancer at the time of this study. Lactating nipple fluids contained a significant amount of bFGF (1065 ng/L [251]).

We also measured the concentrations of another potent angiogenic factor—VEGF (vascular endothelial growth factor)—in nipple fluids, and found no significant differences between the three groups: 106 500 ng/L [19 000] in controls; 92 400 ng/L [19 100] in women with cancer; and 46 100 [17 800] ng/L in women who were lactating. We conclude that VEGF in nipple fluids would not be useful in diagnosing breast cancer.

Patients' nipple fluid has not been extensively investigated as a possible source for diagnostic purposes. Previous studies with carcinoembryonic antigen and prostate specific antigen in nipple fluid showed significant overlap between the study groups.^{4,5} Measurement of bFGF in nipple fluid has the potential to be a useful diagnostic tool for breast cancer. Our results are preliminary, and a larger study is warranted to test this hypothesis.

We thank Judah Folkman for his insightful scientific advice and support, and Polly Candela, Jianbo He, and Seung-Hye Kim for their assistance in data collection. This study was supported by the NIH CA 89433, the Susan Komen Breast Cancer Foundation, the Gonda Foundation, and the Jonsson Comprehensive Cancer Center, DOD grant BC 990959.

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Controls	Lactating	Breast cancer
190	1670	7470
ND	1150	2490
ND	990	2390
ND	450	2240
ND	..	860
ND	..	670
ND	..	590
ND	..	310
ND	..	150
ND	..	ND

ND=not detected.

Nipple fluid bFGF (ng/L)

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January 26, 2000

Ms. Linda S. Stevenson
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Re: UCLA Reference: LA99-547-2
G&C Reference: 30435.72-US-U1
U.S. Patent Application for:
COMPOSITIONS AND METHODS FOR INTRADUCTAL GENE THERAPY

Inventor(s): Sanford H. Barsky and Mary L. Alpaugh

Dear Linda:

Enclosed please find copies of the transmittal papers and the above-identified patent application (unsigned), which were filed in the U.S. Patent and Trademark Office (PTO) on January 20, 2000.

Dr. Barsky was ill and only able to get the new disclosure to me the day before this Application had to be filed. Consequently I was not able to get all of the formal documents signed in time for this filing. Therefore, I will file these documents in response to the Notice of Missing Parts that the Patent Office will issue within the next month or so. At that time the basic filing fee for a small entity (which was deferred in this filing) will also be paid.

If you have any questions or comments regarding this matter, please feel free to contact me at any time.

Very truly yours,



William J. Wood

WJW/amb
Encl.

cc/enc: Dr. Sanford Barsky
C. Patrick Machado, Vice President - Windy Hill Technology, Inc.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): SANFORD H. BARSKY and MARY L. ALPAUGH
 Docket: 30435.72USU1
 Title: COMPOSITIONS AND METHODS FOR INTRADUCTAL GENE THEREAPY

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): SANFORD H. BARSKY and MARY L. ALPAUGH
 Docket: 30435.72USU1
 Title: COMPOSITIONS AND METHODS FOR INTRADUCTAL GENE THEREAPY

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(PTO TRANSMITTAL - NEW FILING)

COMPOSITIONS AND METHODS FOR INTRADUCTAL GENE THERAPY

This application claims the benefit of United States provisional patent application serial number 60/116,470, filed January 20, 1999. The entire contents of this provisional patent application is incorporated by reference into this application.

5 This invention was made with Government support under U.C. Biostar Biotechnology grant S98-42, grant No. BC990959, awarded by the Department of Defense Breast Cancer Research Program and grant No. DAMD 17-96-C-6117, awarded by the Department of the Army, U.S. Army Medical Research Acquisition Activity. The government has certain rights in this invention.

Field of the Invention

10 The present invention relates to the use of cell specific vectors in the diagnosis as well as the prophylactic and therapeutic treatment of diseases of the breast, in particular cancer.

Background of the Invention

15 Cancers of the breast are one of the leading causes of death among women, with the cumulative lifetime risk of a woman developing breast cancer estimated to be 1 in 9. Consequently, understanding the origins of these malignancies as well as the identification of new therapeutic modalities is of significant interest to health care professionals.

20 The mature human breast comprises from six to nine major ducts, which emanate from the nipple, serially branch into ducts and terminate in lobuloalveolar structures (Russo et al., Lab. Invest. 62(3): 244-278 (1990)). The branching network of ducts is composed of luminal epithelial cells in a supporting matrix of connective tissue and myoepithelial cells. Tissues removed from the human female breast during surgery and autopsy have been examined in numerous studies directed at the nature and site of origin of neoplastic growth. Subgross sampling and histological confirmation have enabled pathological characterization of entire breasts which shows that human breast cancer arise within the ductal system of the breast exclusively from luminal epithelial cells. Ductal origin is supported by the presence of more extensive epithelial proliferations, which are presumed to be preneoplastic, in surgically removed cancerous breasts as compared to nonmalignant breasts removed during autopsies.

25 With the significant cumulative lifetime risk of a woman developing breast cancer, there is an urgent need to develop both therapeutic methods of treatment that are more effective, less invasive and accompanied by fewer side effects as well as prophylactic methods of treatment that are more effective

30

than increased and intensified physical monitoring and far less extreme than radical mastectomy. In spite of the recent discovery of the heritable breast cancer susceptibility loci including BRCA1 and other genes (see e.g. Miki et al., Science 266:66-71 (1994)), and the increasing ability of physicians to identify women with elevated breast cancer risk, prophylactic methods are still currently limited to physical monitoring and prophylactic mastectomy.

In view of the above, what is needed in the art is the identification of novel methods which aid in the prevention and treatment of cancers of the mammary gland. In this context, optimal methods are those which have a wide application both in the diagnosis of cancer, as well as the prophylactic and therapeutic treatment of cancer.

Summary of the Invention

The present invention is based on the observations of the respective roles of the epithelial and myoepithelial cell lineages in the development of breast cancer and the fact that these cell types differentially express gene products that can be targeted by cell specific vectors. In this context, new strategies emerge which can be used as a means of breast carcinoma diagnosis, prophylaxis and treatment. A first strategy is to selectively target cells of the breast epithelium so that breast carcinoma does not develop. A second strategy is to target myoepithelial cells as a means of bolstering the myoepithelial defense so that even if DCIS develops, it will be confined to the ductal system indefinitely.

The present invention provides prophylactic and therapeutic methods of treating a disease of the ductal epithelium of the mammary gland, in particular cancer. The present invention further provides diagnostic methods of assessing the status of cancers of the mammary gland. In this context, the present invention provides methods directed to selectively transducing a cell in a ductal system in a mammary gland comprising the step of using ductal cannulation to contact the cell with a vector that selectively targets that cell. In an illustrative embodiment, the invention consists of a method of selectively transducing either a epithelial cell or a myoepithelial cell, by contacting the cell with a vector that selectively targets a CAR molecule that is expressed on the epithelial cell or a heparin sulfate proteoglycan molecule that is expressed on the myoepithelial cell.

In a specific embodiment of the invention, the vector is a replication defective adenovirus which targets a molecule expressed solely by a epithelial cell and subsequently induces cell death by delivery of an cytolysis inducing gene such as thymidine kinase or cytosine deaminase. In a more preferred embodiment, the vector is a replication-competent lytic adenovirus which targets a

coxsackievirus and adenovirus receptor (CAR) molecule expressed by the epithelial cell and subsequently induces cell death by lysis. In a highly preferred embodiment, the replication-competent lytic adenovirus contains a *cis* element such as a lactoalbumin promoter and the MUC1 promoter which stimulates adenovirus replication in the presence of a *trans* factor present in the epithelial cell and induces more effective lysis. The myoepithelial cells of the breast duct, which lack CAR expression (as shown, for example by RT-PCR), are completely resistant to adenovirus infection (transduction) and serve as a barrier to systemic infection.

In another specific embodiment of the invention, the vector is a recombinant adeno-associated virus which targets a molecule expressed by a myoepithelial cell and comprises a polynucleotide which encodes a polypeptide which inhibits the development of epithelial cell cancer. In a specific embodiment, the polypeptide of the recombinant adeno-associated virus inhibits angiogenesis or the proliferation, invasion or metastases of a epithelial cell. In a specific embodiment, the polypeptide is maspin, thrombospondin-1, TIMP-1, protease nexin-II, α -1 antitrypsin or soluble bFGF receptor. In a more preferred embodiment, the recombinant adeno-associated virus contains a *cis* element such as a lactoalbumin promoter and the MUC1 promoter which stimulates recombinant adeno-associated virus replication in the presence of a *trans* factor present in the epithelial cell.

The present invention also provides a method of treating the ductal epithelium of a mammary gland prophylactically for cancer, which method comprises the step of contacting, by ductal cannulation, the ductal epithelium of the mammary gland with a tissue specific vector so as to inhibit the formation of cancer of ductal epithelial origin. In addition, the present invention provides combined therapeutic/prophylactic methods of treating the mammary gland therapeutically by surgery, radiation and/or chemotherapy and, either concomitantly or subsequently, contacting the ductal epithelium of the mammary gland with a cell specific vector which specifically targets a epithelial or a myoepithelial cell.

The present invention also provides a method of determining the lineage of a cell in a ductal system in a mammary gland selected from the group consisting of a luminal epithelial cell and a myoepithelial cell, by using ductal cannulation to contact the cell with vector containing a reporter gene, wherein the vector selectively targets a molecule that is expressed on the epithelial cell or the myoepithelial cell.

In addition, the present invention provides compositions including recombinant adenovirus and recombinant adeno-associated virus vectors as well as myoepithelial cell lines and transplantable xenografts.

Brief Description of the Drawings

Figure 1 is a schematic which depicts the ductal-lobular unit of the breast and illustrates the possibility of gene therapy strategies targeting either epithelial cells and/or myoepithelial cells.

5 Figures 2A is a photograph illustrating the feasibility of gaining access to the entire ductal system of the breast through nipple duct identification and cannulation.

Figure 2B, a photograph showing that when nipple ducts are individually cannulated, an injected dye reaches every ductal orifice.

10 Figure 3A is a photograph showing the absence of β -galactosidase expression in myoepithelial cells contacted with a β -galactosidase containing recombinant adenovirus (Ad2) to illustrate how the expression of CAR on the surface of epithelial cells in the ductal-lobular unit of the breast allows the selective targeting of the epithelial cells and how the absence of CAR on myoepithelial cells confers resistance of this cell to adenoviral infection.

15 Figure 3B is a photograph showing the intense β -galactosidase expression in epithelial cells contacted with a β -galactosidase containing recombinant adenovirus as an illustration of how the expression of CAR on the surface of epithelial cells in the ductal-lobular unit of the breast allows the selective targeting of epithelial cells.

Figure 3C is a photograph showing how CAR expression is completely absent in myoepithelial cells (lane under CAR) but present in ductal epithelial cells and carcinoma lines (other lanes).

20 Figure 4A is a photograph showing reporter gene expression in myoepithelial cells contacted with a recombinant adeno-associated virus containing a human green fluorescence reporter gene which illustrates how the expression of heparin sulfate proteoglycan on the surface of myoepithelial cells in the ductal-lobular unit of the breast allows the selective targeting of the myoepithelial cells.

25 Figure 4B is a photograph showing only background reporter gene expression in epithelial cells contacted with a recombinant adeno-associated virus containing a human green fluorescence reporter gene which illustrates how the expression of heparin sulfate proteoglycan on the surface of myoepithelial cells in the ductal-lobular unit of the breast allows the selective targeting of the myoepithelial cells.

Figure 5 is a Southern blot of DNA cut with Hinf I and probed using the multi-locus Jefferys probe (33.6) showing the distinct and identifying pattern of a number of illustrative myoepithelial cell lines and xenografts disclosed herein (HMS-1, HMS-X, HMS-3, HMS-3X, HMS-4X). All of the myoepithelial cell lines and xenografts exhibited the same susceptibility to recombinant adeno-virus associated virus transfection and the same resistance to adenoviral transfection. Other myoepithelial cell lines and xenografts (HMS-5X, HMS-6X, HMS-5, HMS-6) not depicted exhibit the same properties.

Figure 6 is a Southern blot of DNA cut with Hae III and probed using the multi-locus Jefferys probe (33.6) showing the distinct and identifying pattern of a number of illustrative myoepithelial cell lines and xenografts disclosed herein (HMS-1, HMS-X, HMS-3, HMS-3X, HMS-4X). All of the myoepithelial cell lines and xenografts exhibited the same susceptibility to recombinant adeno-virus associated virus transfection and the same resistance to adenoviral transfection. Other myoepithelial cell lines and xenografts (HMS-5X, HMS-6X, HMS-5, HMS-6) not depicted exhibit the same properties.

Figure 7 is a bar graph comparing MDA-MB-231 breast carcinoma cell invasion in Matrigel in the presence of no myoepithelial cells (control), HMS myoepithelial cells (HMS), rAAV transfected HMS myoepithelial cells (rAAV-HMS) and rAAV-recombinant maspin transfected HMS myoepithelial cells (rAAV-maspin-HMS). The results demonstrate that myoepithelial cells which overexpress maspin are highly effective at blocking carcinoma cell invasion in Matrigel. Specifically, the effects of transfected myoepithelial clones expressing recombinant maspin in invasion inhibition assays show a 200% increase in inhibition of invasion.

Detailed Description of the Invention

Definitions:

The term "transduce" is used in its broadest sense and refers to a process wherein a vector gains entry in to a cell so that polynucleotides of the vector are delivered to the cell.

5 The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. eucaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

10 Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences
15 being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking may be accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers may be used in accordance with conventional practice.

"Polynucleotide" and "nucleic acid" refer to single- or double-stranded molecules which may be
20 DNA, comprised of the nucleotide bases A, T, C and G, or RNA, comprised of the bases A, U (substitutes for T), C, and G. The polynucleotide may represent a coding strand or its complement. Polynucleotide molecules may be identical in sequence to the sequence which is naturally occurring or may include alternative codons which encode the same amino acid as that which is found in the naturally occurring sequence (See, Lewin "Genes V" Oxford University Press Chapter 7, pp. 171-174
25 (1994)). Furthermore, polynucleotide molecules may include codons which represent conservative substitutions of amino acids as described. The polynucleotide may represent genomic DNA or cDNA.

"Polypeptide" refers to a molecule comprised of amino acids which correspond to those encoded by a polynucleotide sequence which is naturally occurring. The polypeptide may include conservative substitutions where the naturally occurring amino acid is replaced by one having similar
30 properties, where such conservative substitutions do not alter the function of the polypeptide (See, Lewin "Genes V" Oxford University Press Chapter 1, pp.: 9-13 (1994)).

The gene therapy strategies provided herein are predicated on either selectively destroying the breast epithelial cell (where cancers arise) and/or enhancing the functions of the myoepithelial cell (which are natural cancer suppressors). The present invention utilizes observations of the different of the ductal epithelial and ductal myoepithelial cell lineages in the development of breast cancer and the fact that these cell types differentially express gene products that can be targeted by cell specific vectors. In this context, new strategies emerge which can be used as a means of breast carcinoma diagnosis, prophylaxis and treatment. A first strategy is to selectively transduce luminal epithelium cells so that breast carcinoma does not develop. A second strategy is to selectively transduce myoepithelial cells as a means of bolstering the myoepithelial defense so that even if DCIS develops, it will be confined to the ductal system indefinitely.

The present invention takes advantage of observations that early breast cancer can be thought of as a disease of the ductal system which arises from ductal epithelium and that human breast cancer arises within the ductal system of the breast exclusively from epithelial cells that are located within the breast duct. Throughout this application, the epithelial cells from the breast duct are referred to by a variety of art accepted designations such as ductal epithelial cells, acinar epithelial cells, luminal epithelial cells etc. (see e.g. Virchows et al., Arch. 391(1): 45-51 (1981); Cristov et al., Am. J. Pathol. 138(6): 1371-7 (1991); Lochter, Biochem. Cell Biol. 76(6): 997-1008 (1998); Lakhani et al., J. Pathol. 189(4): 496-503 (1999)) The various designations are used for purposes of clarity in the context in which they are discussed and all refer to epithelial cells which can be selectively transduced by vectors which target CAR expressed by these cells. Preferably, such cells reside within the breast duct system. When cancer is confined by this system it is termed ductal carcinoma *in situ* or DCIS. Recent evidence suggests that the determinants of the progression of DCIS to invasive cancer are epigenetic rather than genetic and consist of strong paracrine regulation by neighboring myoepithelial cells (T. Kuukasjarvi et al. (1997) Am. J. Pathol., 150:1465-1471). Myoepithelial cells are cells which surround this ductal system and keep the developing cancer termed ductal carcinoma *in situ* (DCIS) confined. Because of their close proximity, myoepithelial cells would be anticipated to exert important paracrine influences on DCIS progression. Myoepithelial cells accomplish this by their production and secretion of a number of proteinase inhibitors.

Myoepithelial cells of the breast differ from luminal ductal and acinar epithelial cells in many ways: they lack expression of the common hormonal receptor, ER-I, and its responsive genes like PR; they lie next to the basement membrane and contribute to the synthesis of that structure; they rarely transform or proliferate and when they do give rise to only low grade benign neoplasms (M.D.

Sternlicht and S.H. Barsky (1997) Medical Hypotheses, 48:37-46). Myoepithelial cells are ubiquitously present in normal ducts, benign proliferations such as adenosis and precancerous proliferations, e.g. DCIS. These cumulative studies suggest that DCIS is cancer of the breast in the true genetic, biological and clinical sense of the word but is limited within the confines of the ductal system by myoepithelial cells. Both epithelial cells and myoepithelial cells can then be targets of intraductal gene therapy provided that specific vectors can be identified which will selectively target each cell type.

The findings disclosed herein allow specific gene therapy approaches to carry out the strategies discussed above. A first finding is that it is possible to gain access to the entire ductal system of the breast through nipple duct identification and cannulation. A second finding is that primary mammary ductal epithelial cells express the coxsackievirus-Ad receptor (CAR, see e.g. J. Bergelson et al. (1997) Science 275:1320-1323) and are easily transduced with vectors that gain entry to the cell via CAR mediated uptake (such as certain adenovirus groups) whereas myoepithelial cells lack CAR and are completely resistant to transduction by vectors which target this molecule. As illustrated in Table I below, a third finding is that myoepithelial cells, do not express CAR and instead express cell surface heparin sulfate proteoglycan (see e.g. Summerford et al., J.Virol. 72(2), 1438-1445 (1998)) and are easily transduced with vectors that gain entry to the cell via heparin sulfate proteoglycan mediated uptake such as rAAV) whereas primary ductal epithelial cells do not express this proteoglycan and are resistant to transduction by vectors which target this molecule. These findings are supported by a number of experiments with established myoepithelial xenografts and cell lines (both immortalized and in limited short term passage), which further demonstrate the selectiveness of the rAAV for the myoepithelial target.

Table 1 depicts high cell surface heparin sulfate proteoglycan content of myoepithelial cells (HMS-1)

	Uronic Acid ^b		Glycosaminoglycan Content (%)				
	(dry wt)	(DNA)	CS	HA	HS	DS	KS
Normal salivary gland	1.83	0.031	4	56	24	16	0
Basal cell adenoca (HMS-1)	0.63	0.008	32	12	24	0	32
Basal cell adenoca (HMS-X)	10.45	0.530	78	12	8	2	0
Adenoidcystic Ca (HMS-3X)	8.96	0.298	77	11	9	3	0
Pleomorphic adenoma	26.49	3.718	89	7	0	4	0
Squamous cell Ca (A253)	0.77	0.009	19	59	14	8	0
Squamous cell Ca (A253-X)	3.93	0.094	30	27	31	12	0
Murine EHS tumor	7.62	0.356	0	6	94	0	0
Cartilage	ND	ND	61	15	3	21	0

All data represent mean values obtained from two or more determinations from the same sample. Uronic acid was measured in micrograms per milligram of tissue (dry weight) and micrograms per microgram of DNA. CS, chondroitin sulfate; HA, hyaluronic acid; HS, heparin sulfate; DS, dermatan sulfate; KS, keratin sulfate; not determined.

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The findings disclosed herein illustrate the feasibility of, using an intraductal approach and different cell specific viral vectors such as rAd, selectively targeting and destroying the breast epithelium *in vivo* while sparing the underlying myoepithelium. Further, these findings illustrate the feasibility of, using an intraductal approach and different viral vectors such as rAAV, selectively targeting the myoepithelium *in vivo* with a vector containing a molecule capable of inhibiting epithelial luminal cell growth and invasion as a means of bolstering the suppressive effects of these cells. In addition, these findings allow for the utilization of replication-defective rAd and rAAV containing reporter genes in diagnostic studies which evaluate the expansion of a specific cell type. While various embodiments of the methods disclosed herein can be used to treat any exocrine gland, they are particularly useful in the treatment of the mammary gland.

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The methods disclosed herein overcome a number of limitations of methods known in the art. Specifically, while a number of vectors (e.g. vaccinia, sindbis and certain adenoviral and retroviral vectors) exhibit a wide tissue tropism and are used to transduce a variety cell lineages including epithelial and myoepithelial cells, the broad specificity of these vectors can also limit their usefulness. In particular, while vectors having a wide tissue tropism are favored in certain contexts, this property is disadvantageous in situations where it is desirable to selectively transduce a specific cell lineage that is present in a mixed population of different cell lineages. For example, practitioners using vectors having a wide tissue tropism *in vivo* may be forced to use them only in contexts where a host's rapid immunological response will prevent them from escaping the milieu in to which they are introduced.

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The vectors and methods disclosed herein overcome such problems by allowing the artisan to selectively transduce a specific lineage (such as epithelial or myoepithelial cells) that is present within a mixed population of cells of different lineages. Specifically, methods utilizing vectors which selectively transduce a specific lineage restrict the ability of such vectors to gain entry into proximal cells of different lineages (which are not transduced by the vector), and consequently inhibit such vectors from escaping the milieu in to which they are introduced.

Vectors which Selectively Transduce Epithelial Cells

A significant feature of the methods of the invention disclosed herein is the identification and utilization cell specific vectors which have the ability to selectively transduce epithelial cells and not transduce myoepithelial cells. Specifically, as these vectors gain entry into target cells via a molecule which is expressed on epithelial but not myoepithelial cells, they can be used to selectively transduce epithelial cells within a mixed population of epithelial and myoepithelial cells.

A variety different vectors which gain entry into cells through the CAR molecule can be used to selectively transduce epithelial cells. Strains of the coxsackie B viruses (such as coxsackie B3 and B4) for example enter cells via CAR mediated uptake (see e.g. Bergelson et al., J.Virol. 72(1), 415-419 (1998)). Therefore, coxsackie virus vectors can be used to selectively transduce an epithelial cell in a ductal system in a mammary gland that expresses CAR molecules. In addition, certain subgroups of adenovirus also gain entry into cells via CAR. In particular, adenovirus serotypes from subgroups A (e.g. Ad12), C (e.g. Ad1, Ad2, Ad5 and Ad6), D (e.g. Ad9, Ad15, Ad30 and Ad37), E (e.g. Ad4), and F (e.g. Ad40 and Ad41) all use CAR as a cellular fiber receptor (see e.g. Roelvink et al., J. Virol. 72(10): 7909-7915 (1998)). It is however important to note that adenoviruses can target different molecules expressed by target cells and that not all adenovirus strains can be used to selectively transduce CAR expressing epithelial cells (see e.g. Nalbantoglu et al., Hum. Gene Ther. 10(6): 1009-1019 (1999). For example, subgroup B adenoviruses (e.g. Ad3, Ad7 and Ad35) appear to interact with target cells through a different cellular receptor than CAR and may not function in the disclosed methods (see e.g. Stevenson et al., J. Virol. 69(5): 2850-2857 (1995)). Therefore it is important to establish that any vector (adenovirus or otherwise) used in the disclosed methods can selectively transduce epithelial cells while not transducing proximal myoepithelial cells.

In addition to vectors having a natural tropism for CAR, it is known in the art that a wide variety of vectors may be constructed to target a specific molecule on a cell such as CAR. In particular, target cell specificity of delivery vectors can be provided by incorporation of a target cell specific binding domain by the use of any binding domain, which binds specifically to a binding site on the

target cell (see e.g. U.S. Patent No. 5,834,589 incorporated herein by reference). Because the CAR binding adenoviral fiber protein residues have been identified (see e.g. Santis et al., J. Gen. Virol. 80(Pt 6): 1519-1527 (1999)) and because the adenovirus binding activity of CAR has been localized to the amino-terminal IgV-related domain of this molecule, one skilled in the art generate target vectors specific for this binding domain.

As noted above, certain viral vectors may be able to gain entry in to cells through multiple receptors expressed on the surface of a cell. For example, certain adenoviral subtypes may be able to gain entry in to cells via the MHC class I $\alpha 2$ domain or members of the $\beta 2$ integrin family (see e.g. Davison et al., J. Virol. 73(5): 4513-4517 (1999) and Huang et al. J. Virol. 70(7): 4502-4508 (1996)). Therefore, it is important to establish that any vector (adenovirus or otherwise) used to selectively transduce epithelial cells will not transduce proximal myoepithelial cells. In particular, as certain vectors may be able to gain entry into a target cell by more than one receptor, it is prudent to assess the cellular specificity of any candidate vectors. Methods for assessing the specificity of a candidate vector are well known in the art and consist merely of contacting a target cell of interest with a candidate vector and observing if transduction occurs. In this context, Figure 3 provides an illustrative example of an assessment of the adenoviral vector used in the disclosed examples. In particular, Figure 3A shows the absence of β -galactosidase expression in myoepithelial cells contacted with a β -galactosidase containing recombinant adenovirus to illustrate how the expression of CAR on the surface of epithelial cells allows the selective targeting of the epithelial cells and how the absence of CAR on myoepithelial cells confers resistance of this cell to adenoviral infection. In contrast, Figure 3B shows the intense β -galactosidase expression in epithelial cells contacted with a β -galactosidase containing recombinant adenovirus as an illustration of how the expression of CAR on the surface of epithelial cells allows the selective transduction of epithelial cells.

Vectors which Selectively Transduce Myoepithelial Cells

A significant feature of the methods of the invention disclosed herein is the identification and utilization of cell specific vectors which have the ability to selectively transduce myoepithelial cells and not transduce epithelial cells. Specifically, as these vectors gain entry into target cells via a molecule which is expressed on myoepithelial but not epithelial cells, they can be used to selectively transduce myoepithelial cells within a mixed population of myoepithelial and epithelial cells.

Different vectors which gain entry into cells through the heparin sulfate proteoglycan molecule can be used to selectively transduce myoepithelial cells. The human parvovirus, Adeno-associated

virus-2 (AAV) for example can enter cells via heparin sulfate proteoglycan mediated uptake (see e.g. C. Summerford and R.J. Samulski, *J. Virology* 72:1438-1445)). In addition, herpesviruses are believed to target cells through their heparin sulfate proteoglycan molecules (see e.g. Zhu et al., *P.N.A.S.* 92(8): 3546-3550 (1995)). It is however important to note that viral vectors can attach different molecules expressed by target cells and/or may utilize co-receptors to gain entry in to a cell (see e.g. Summerford et al., *Nat. Med.* 5(1): 78-82 (1999) and Qing et al., *Nat. Med.* 5(1): 71-11 (1999)). Therefore it is important to establish that any vector (adeno-associated virus-2 or otherwise) used in the disclosed methods can selectively transduce myoepithelial cells while not transducing proximal epithelial cells.

In addition to vectors having a natural tropism for heparin sulfate proteoglycan, it is known in the art that a wide variety of vectors may be constructed to target a specific molecule on a cell such as heparin sulfate proteoglycan. In particular, target cell specificity of delivery vectors can be provided by incorporation of a target cell specific binding domain by the use of any binding domain, which binds specifically to a binding site on the target cell (see e.g. U.S. Patent No. 5,834,589 incorporated herein by reference).

As noted above, certain viral vectors may be able to gain entry in to cells through multiple receptors expressed on the surface of a cell. For example, adeno-associated virus 2 may be able to gain entry in to cells via the human fibroblast growth factor receptor 1 or $\alpha V\beta$ integrin (see e.g. Summerford et al., *Nat. Med.* 5(1): 78-82 (1999) and Qing et al., *Nat. Med.* 5(1): 71-11 (1999)). Therefore, it is important to establish that any vector (adeno-associated virus 2 or otherwise) used to selectively transduce myoepithelial cells will not transduce proximal epithelial cells. In particular, as certain vectors may be able to gain entry into a target cell by more than one receptor, it is prudent to assess the cellular specificity of any candidate vectors. Methods for assessing the specificity of a candidate vector are well known in the art and consist merely of contacting a target cell of interest with a candidate vector and observing if transduction occurs. In this context, Figure 4 provides an illustrative example of an assessment of the Adeno-associated virus-2 used in the disclosed examples. In particular, Figure 4A shows reporter gene expression in myoepithelial cells contacted with a recombinant adeno-associated virus containing a human green fluorescence reporter gene and illustrates how the expression of heparin sulfate proteoglycan on the surface of myoepithelial cells allows the selective targeting of the myoepithelial cells. In comparison, Figure 4B shows only background reporter gene expression in epithelial cells contacted with a recombinant adeno-associated virus containing a human green fluorescence reporter gene and illustrates how the expression of

heparin sulfate proteoglycan on the surface of myoepithelial cells allows the selective transduction of the myoepithelial cells.

Generation and Manipulation of Vectors of the Invention

Methods for generating and manipulating vectors such as the recombinant adeno-associated virus and adenovirus vectors disclosed herein are well known in the art, for example in U.S. Patent Nos. 5,681,731, 5,585,362, 5,756,283 and 5,843,742 which are incorporated herein by reference. As discussed below, a variety of art accepted techniques may be utilized in the practice of embodiments of the invention disclosed herein. While the vectors of the invention may be known in the art such as replication-competent lytic adenoviruses, the vectors can also be manipulated to facilitate their use in the methods disclosed herein. For example, a nucleic acid (eg, cDNA or genomic DNA) containing a regulatory region such as a promoter or an enhancer may be inserted into a cell specific vector for cloning (amplification of the DNA) or for expression. Alternatively, a nucleic acid encoding a gene of interest such as a suicide gene or tumor suppressive gene may be inserted into a cell specific vector for expression in a target cell.

The nucleic acid sequence of interest may be inserted into a vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

Both expression and cloning vectors can contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of mammalian, bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2: plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, eg, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, eg, the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up a nucleic acid of interest, such as Neomycin, DHFR or

thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., *Nature*, 282:39 (1979); Kingsman et al., *Gene*, 7:141 (1979);
5 Tschemper et al., *Gene*, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, *Genetics*, 85:12 (1977)].

Expression and cloning vectors can contain a promoter operably linked to the nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well
10 known. Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems [Chang et al., *Nature*, 275:615 (1978); Goeddel et al., *Nature*, 281:544 (1979)], alkaline phosphatase, a tryptophan (*trp*) promoter system [Goeddel, *Nucleic Acids Res.*, 8:4057 (1980); EP 36,776], and hybrid promoters such as the *tac* promoter [deBoer et al., *Proc. Natl. Acad. Sci. USA*, 80:21-25 (1983)]. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence
15 operably linked to the DNA encoding a nucleic acid of interest.

Transcription from vectors in mammalian host cells can be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous
20 mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems. In addition, a polynucleotide of interest can be under the transcriptional regulation of an inducible promoter such as the Tet promoter. Alternatively, it can be under the control of an epithelial tissue-specific or cell-specific promoter. Epithelial cell-specific promoters, such as whey acidic protein (*wap*), can be used to
25 target expression of a given gene, e.g., a suicide gene, in ductal epithelial cells. Use can also be made of promoters which control wild-type tumor suppressor genes, such as *Maspin*, *p53* or *Mcs-1* (rat), homeobox genes which are expressed in normal cells but not in cancerous cells.

Transcription of a DNA encoding a polynucleotide of interest by mammalian cells may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of
30 DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eucaryotic cell virus. Examples

include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the coding sequence, but is preferably located at a site 5' from the promoter.

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Methods for Selectively Transducing Epithelial and Myoepithelial Cells

The methods of the invention disclosed herein allow the selective transduction of both ductal epithelial and myoepithelial cells. An illustrative embodiment of the invention is a method of selectively transducing a cell within a mixed population of ductal epithelial and myoepithelial cells, comprising the step of contacting the cell with a cell specific vector that transduces the cell through a molecule that is expressed only on the ductal epithelial cell or, alternatively, through a different molecule that is expressed only on the myoepithelial cell. In a preferred embodiment of this invention, the mixed population of ductal epithelial and myoepithelial cells is within a ductal system of a mammary gland and the cells to be transduced are contacted with the vector by ductal cannulation.

15 In a specific embodiment of this invention, cell is a ductal epithelial cell that is transduced with a vector (such as a replication competent adenovirus) that induces cell death. In a related embodiment, the vector can contain a suicide gene that can induce cell death such as thymidine kinase or cytosine deaminase. In another specific embodiment of the invention, the vector contains a *cis* element which stimulates viral replication or specific gene expression in the presence of a *trans* factor present in the ductal epithelial cell. For example, the *cis* element could be one of the large number of known regulatory sequences such as the lactoalbumin promoter and the MUC1 promoter.

20 In yet another preferred embodiment of this invention, the cell is a myoepithelial cell that is transduced with a vector (such as a recombinant adeno-associated virus) that comprises a gene encoding a polypeptide which inhibits the development of cancer. In a specific embodiment of this invention, the vector contains a *cis* element contains a *cis* element which modulates the expression of the gene in the presence of a *trans* factor present in the myoepithelial cell. In preferred embodiments of this method, the polypeptide inhibits the proliferation of a ductal epithelial cell, the invasion of a ductal epithelial cell, endothelial migration, angiogenesis or increases the production of nitric oxide by the myoepithelial cell. Illustrative polypeptides include maspin, thrombospondin-1, TIMP-1, protease nexin-II, α -1 antitrypsin and soluble bFGF receptor. Preferably the polypeptide induces death (for example by apoptosis) of a ductal epithelial cell.

In a related embodiment, the invention consists of a method of selectively transducing a cell in

a ductal system in a mammary gland selected from the group consisting of a ductal epithelial cell and a myoepithelial cell, comprising the step of contacting, by ductal cannulation, the cell with a vector that selectively targets a CAR molecule that is expressed on the ductal epithelial cell or a heparin sulfate proteoglycan molecule that is expressed on the myoepithelial cell. In yet another related embodiment, the invention consists of a method of selectively transducing a ductal epithelial cell in a ductal system in a mammary gland, comprising the step of contacting, by ductal cannulation, the cell with a vector that targets a CAR molecule expressed by the cell. In yet another related embodiment, the invention consists of a method of selectively transducing a myoepithelial cell in a ductal system in a mammary gland, comprising the step of contacting, by ductal cannulation, the cell with a vector that targets a heparin sulfate proteoglycan molecule expressed by the cell.

In a related embodiment, the invention is a method of transducing ductal epithelial cells within a mixed population of ductal epithelial and myoepithelial cells, while not transducing proximal myoepithelial cells, by contacting the ductal epithelial cells with a vector that gains entry into ductal epithelial cells via a CAR molecule expressed by the ductal epithelial cells. In yet another related embodiment, the invention consists of a method of transducing ductal epithelial cells within a mixed population of ductal epithelial and myoepithelial cells, wherein the myoepithelial cells are not transduced, by contacting the ductal epithelial cells with a vector that gains entry into the ductal epithelial cells via a CAR molecule expressed by the ductal epithelial cells but not expressed by the myoepithelial cells.

In yet another related embodiment, the invention consists of a method of transducing myoepithelial cells within a mixed population of ductal epithelial and myoepithelial cells, while not transducing proximal ductal epithelial cells, by contacting the myoepithelial cells with a vector that gains entry into myoepithelial cells via a heparin sulfate proteoglycan molecule expressed by the myoepithelial cells. In yet another related embodiment, the invention consists of a method of transducing myoepithelial cells within a mixed population of ductal epithelial and myoepithelial cells, wherein the ductal epithelial cells are not transduced, by contacting the myoepithelial cells with a vector that gains entry into the myoepithelial cells via a heparin sulfate proteoglycan molecule expressed by the myoepithelial cells but not expressed by the ductal epithelial cells.

Prophylactic Methods for Treating a Breast Duct

The prophylactic methods of the present invention involve methods of selectively treating a cell in the duct of a breast prophylactically for a disease that affects the ductal epithelium of the breast such

as cancer. One embodiment of these methods consists of selectively transducing either a luminal epithelial cell, a myoepithelial cell (or both a luminal epithelial cell and a myoepithelial cell) within the duct of a breast, by using ductal cannulation to contacting the cell of interest with a vector that selectively targets a molecule that is expressed on either the luminal epithelial cell or the myoepithelial cell. Depending upon which cell is contacted, the vectors used in these methods are then used to effect a desired biological activity such as cytolysis or the expression of a soluble effector molecule.

In an exemplary embodiment, the method comprises contacting, preferably by ductal cannulation, a myoepithelial or epithelial cell with a cell specific vector that either suppresses the growth of cancerous cells or effects the destruction of all or less than all of the ductal epithelium so as to inhibit the formation of cancer of ductal epithelial origin. In another illustrative embodiment, the invention consists of a method of treating the ductal epithelium of a mammary gland for cancer of ductal epithelial cell origin, comprising the step of contacting, by ductal cannulation, a cell in a ductal system in a mammary gland selected from the group consisting of a ductal epithelial cell and a myoepithelial cell, with a vector that enters the cell through a CAR molecule expressed by the ductal epithelial cell or a heparin sulfate proteoglycan molecule expressed by the myoepithelial cell, wherein the expression of the polynucleotides of the vector so transduced inhibits the formation of cancer of ductal epithelial cell origin.

In a specific embodiment of the prophylactic methods of the present invention, epithelial cells of the mammary gland are treated prophylactically with a cytotoxic cell specific vector so as to inhibit the formation of cancer of ductal epithelial origin. In this context, only those cytolytic viruses which exhibit the proper cell specificity (i.e. those which selectively transduce epithelial cells via the CAR molecule expressed by these cells) can be used in the methods of the invention. In another specific embodiment of the prophylactic methods of the present invention, myoepithelial cells of the mammary gland are treated prophylactically with a cell specific vector comprising a polynucleotide encoding a suppressor molecule so as to inhibit the formation of cancer of ductal epithelial origin.

The prophylactic methods of treating a mammary gland described herein are particularly useful in treating a mammary gland in a mammal at risk for developing breast cancer. The mammary gland can be characterized as one that has never had a tumor, one that had a tumor previously but the tumor is no longer detectable due to other prior therapeutic treatment, or one that has an incipient or occult tumor, preneoplasia or ductal hyperplasia. Normally, hyperplasias and incipient and occult tumors are not detectable by means of physical examination or radiology. Accordingly, the prophylactic method will find use in cases where there is reason to take some prophylactic measures, such as when there are

known inherited factors predisposing to cancers, where there are suspicious lesions present in a breast with the potential for developing into a malignancy, where there has been exposure to carcinogenic agents in the environment, where age predisposes to a cancer, where cancer of another gland, e.g. the mammary gland of the contralateral breast, suggests a propensity for developing cancer, or where there is a fear or suspicion of metastasis.

The methods of the present invention can also be combined with other methods of prophylactic and therapeutic treatment in addition to those cited herein, such as methods that target destruction of cancer cells, e.g., by targeting of cell-surface markers, receptor ligands, e.g., ligands to gastrin-releasing peptide-like receptors, tumor-associated antigens, e.g., the 57 kD cytokeratin or the antigen recognized by the monoclonal antibody GB24, the extracellular matrix glycoprotein tenascin, antisense oncogenes such as c-fos, homeobox genes that are expressed in cancer cells but not normal cells, tumor-infiltrating lymphocytes that express cytokines, RGD-containing peptides and proteins, which are administered following surgery, lipophilic drug-containing liposomes to which are covalently conjugated monoclonal antibodies for targeting to cancer cells, low fat diet, moderate physical exercise and hormonal modulation.

Introduction of Vectors via Ductal Cannulation

The ductal epithelium is preferably contacted with the agent by introduction of the agent through the duct of the exocrine ductal epithelium, such as by ductal cannulation. In the mammary gland, there are 6-9 major ducts that emanate from the nipple and serially branch into other ducts, terminating in lobulo-alveolar structures (Russo et al. (1990), supra). Accordingly, in some circumstances, such as those in which even more localized treatment is necessary or desired, for example, by the choice of anti-cancer agent, it may be preferable to contact the ductal epithelium of the exocrine gland through one of these major ducts connecting to a lobulo-alveolar structure. In this regard, ductal cannulation enables intratumoral injection.

Methods of ductal cannulation are known in the art (see e.g. S.M. Love and S.H. Barsky (1996) The Lancet, 348:997-999; Makita et al., Breast Cancer Res. Treat. 18: 179-188 (1991) and Okazaki et al., Jpn J Clin Oncol 21: 188-193 (1991)). Briefly, a breast can be prepared and draped, with the breast-duct orifices identified with magnifying loupes or the methods described below. One or more ducts can then be cannulated with cannula known in the art such as a rigid metal duct-probe (6 FR Taber-Rothschild Galacrography Kit, Manan Medical Products Inc., Northbrook, Illinois).

In order to facilitate the location individual orifices in a nipple of a breast, one can employ methods for transiently marking and locating individual orifices in a nipple of a breast as further

described in U.S. Serial No. 09/153,564, incorporated herein by reference. Moreover, methods for gaining access and evaluating cells of a breast duct as further described in U.S. Serial Nos. 09/067,661 and 09/301,058, incorporated herein by reference. For example, one can introduce a detectable substance, such as a labeling reagent, dye, or the like, to the nipple so that the substance localizes and/or accumulates at or near the orifice to permit visual, automated, or other detection. Alternatively, one can utilize other stimuli for inducing a response, change, or reaction at or near a location of the orifice in the nipple. For example, it may be possible to illuminate the nipple with certain light or other energies which help distinguish between the orifice and other tissue surfaces. It may also be possible to introduce chemical reagents which react with ductal secretions at the orifice to enhance visibility e.g. to produce a visible or otherwise detectable reaction product.

Methods Targeting Epithelial Cells

In a specific embodiment of the prophylactic methods of the present invention, luminal epithelial cells in a mammary gland are treated prophylactically for cancer so as to inhibit the formation of cancer of ductal epithelial origin. The method comprises contacting, preferably by ductal cannulation, a luminal epithelial cell in the mammary gland with a vector that selectively targets the luminal epithelial cell. In a preferred embodiment, the vector is a replication competent adenovirus which targets a CAR molecule expressed by the luminal epithelial cell and subsequently induces cell death by lysis. In another embodiment, the vector contains a suicide gene such as thymidine kinase or cytosine deaminase and induces cell death via apoptosis. In a more preferred embodiment, the replication-competent lytic adenovirus contains a *cis* element such as a lactoalbumin promoter and the MUC1 promoter which stimulates adenovirus replication in the presence of a *trans* factor present in the epithelial cell which enhances viral replication, lysis and cell death. With respect to the replication competent adenovirus strategy, the resistance of myoepithelial cells to adenovirus infection inhibits systemic infection by adenovirus and limits the gene and/or viral therapy to the ductal system of the breast.

Other vectors are contemplated in addition the adenovirus viral vectors described herein. For example, coxsackie viruses also target cells through the CAR molecule (see e.g. Bergelson et al., Science 275(5304): 1320-1323 (1997)). Therefore, coxsackie virus vectors can also be used to selectively transduce a cell in a ductal system in a mammary gland that expresses CAR molecules. Moreover, it is known in the art that a wide variety of vectors may be constructed to target a specific molecule, such as CAR, on a cell. In particular, target cell specificity of delivery vectors can be provided by incorporation

of a target cell specific binding domain by the use of any binding domain, which binds specifically to a binding site on the target cell (see e.g. U.S. Patent No. 5,834,589).

As discussed above, a cell specific vector such as the adenoviral vectors disclosed herein can further comprise a suicide gene to enhance the destruction of epithelial cells in the breast. For example, a vector comprising a suicide gene, upon transformation of a epithelial cell and expression therein, renders the transformed cell sensitive to the epithelium-destroying agent, increases the sensitivity of the transformed cell to the agent, converts the agent from a prodrug to an active drug, activates the conversion of the agent from a prodrug to an active drug, enhances the effect of the agent or, itself, produces a protein that is cytotoxic. A preferred suicide gene for use in the present inventive methods is thymidine kinase, such as is found in the Herpes simplex virus, which phosphorylates nucleoside analogues including gancyclovir, which, in turn, inhibits DNA replication. Another example of a suicide gene is cytosine deaminase, which is used in conjunction with 5-fluorocytosine. If the vector comprising the suicide gene is administered locally to the ducts, the cytotoxic agent or precursor can be administered systemically, since only transfected cells will be affected. In this regard, the bystander effect, i.e., the death of neighboring uninfected cells, presumably due to transfer of toxic byproducts through gap junctions between cells in the same compartment, obviates the need for every cell in the ductal epithelium, which is to be destroyed, to be infected. However, sufficient time must be allowed between contacting the epithelial cell with the suicide gene and the prodrug, for example, to achieve efficient killing of the breast epithelial cells.

A cell specific vector comprising an apoptosis-inducing gene also can be used as an agent that destroys a epithelial cell of a breast (Vaux, Cell 76:777-779 (1994)). Examples of apoptosis-inducing genes include ced genes, myc genes (overexpressed), the bclxs gene, the bax gene, and the bak gene. The apoptosis-inducing gene causes death of transfected cells, i.e., by inducing programmed cell death. For example, the bclxs gene, bax gene, or bak gene can be used to inhibit bcl-2 or bcl-X[L], leading to apoptosis. Where necessary, a vector comprising an apoptosis-inducing gene can be used in combination with an agent that inactivates apoptosis inhibitors such as bcl-z, p35, IAP, NAIP, DAD1 and A20 proteins.

Suicide and apoptosis genes can be administered by way of a cell specific vector, such as the adenoviral vectors described in the Examples below. Adenoviral vectors are favored because they enable the generation of high titer recombinant viruses and the efficient transduction of postmitotic cells because adenoviral DNA exists as an episome in the nucleus (Verma, Molecular Medicine 1:2-3 (1994)).

Methods Targeting Myoepithelial Cells

In another embodiment of the prophylactic methods of the present invention, the myoepithelium of a mammary gland can be treated prophylactically for cancer so as to inhibit the formation of cancer of ductal epithelial origin. The method comprises contacting, preferably by ductal cannulation, the myoepithelium of the mammary gland with a vector comprising a molecule which can inhibit the formation of cancer of ductal epithelial origin, for example, maspin. In a preferred embodiment, the vector is a recombinant adeno-associated virus which targets a heparin sulfate proteoglycan molecule expressed by the myoepithelial cell and comprises a polypeptide which inhibits the development of epithelial cell cancer. In a specific embodiment, the polypeptide of the recombinant adeno-associated virus inhibits angiogenesis or the proliferation, invasion or metastases of a luminal epithelial cell. In a specific embodiment, the polypeptide is maspin, thrombospondin-1, TIMP-1, protease nexin-II, α -1 antitrypsin or soluble bFGF receptor.

Other vectors are contemplated in addition the adeno-associated viral vectors described herein. For example, herpesviruses are believed to target cells through their heparin sulfate proteoglycan molecules (see e.g. Zhu et al., P.N.A.S. 92(8): 3546-3550 (1995)). Therefore, herpesvirus vectors such as those known in the art (see e.g. Levatte et al., Neuroscience 86(4): 1321-1336 (1998)), can also be used to selectively transduce a cell in a ductal system in a mammary gland that expresses heparin sulfate proteoglycan molecules. Moreover, it is known in the art that a wide variety of vectors may be constructed to target a specific molecule, such as heparin sulfate proteoglycan, on a cell. In particular, target cell specificity of delivery vectors can be provided by incorporation of a target cell specific binding domain by the use of any binding domain, which binds specifically to a binding site on the target cell (see e.g. U.S. Patent No. 5,834,589).

Therapeutic Methods for Treating a Breast Duct

Embodiments of therapeutic methods of the present invention are related to and can parallel the prophylactic methods described above and include treating the ductal epithelium of a breast therapeutically for a disease that affects the ductal epithelium. In one embodiment of the therapeutic methods of the present invention, the ductal epithelium of a mammary gland is treated therapeutically for cancer so as to destroy cancerous and noncancerous epithelial cells of the ductal epithelium and inhibit the spread of cancer. In an exemplary embodiment, the method comprises contacting, preferably by ductal cannulation, a myoepithelial or epithelial cell with a cell specific vector that can either effect the suppression of the growth of cancerous cells or effect the destruction of all or less than all cancerous cells of ductal epithelial origin. In a specific embodiment of the therapeutic methods of

the present invention, epithelial cells of the mammary gland are contacted with a cytotoxic cell specific vector so as to destroy cancerous cells of ductal epithelial origin. In another specific embodiment of the therapeutic methods of the present invention, myoepithelial cells of the mammary gland are contacted with a cell specific vector comprising a polynucleotide encoding suppressor molecule so as to inhibit the progression of cancers of ductal epithelial origin. In such therapeutic methods, the epithelial-destroying agent should suppress the growth of or destroy all of the diseased or malignant epithelium. In addition, the ductal epithelium immediately surrounding the diseased/malignant epithelium also preferably should be suppressed or destroyed.

Combined Therapeutic/Prophylactic Methods for Treating a Breast Duct

The present invention also provides methods of treating the ductal epithelium of a mammary gland using prophylactic and therapeutic methods known in the art in combination with the prophylactic and therapeutic methods disclosed herein. Examples of such art accepted methods include surgical removal of the cancerous tissue, radiation therapy and chemotherapy. Such combination methods would then comprise an art accepted methods such as the surgical removal of the cancerous tissue and then further contacting, either concomitantly with or subsequently to the therapeutic treatment, the ductal epithelium of the mammary gland, e.g., by ductal cannulation, with the cell specific vectors disclosed herein, so as to suppress or destroy any remaining cancerous cells and noncancerous cells and to inhibit the spread of cancer.

Diagnostic Methods for Evaluating a Breast Duct for Cancer

The present invention further allows for the utilization of cell specific vectors containing reporter genes in diagnostic studies which can evaluate the expansion of a specific cell lineage. Specifically, using the selective transduction methods described herein, one can determine whether a group of cells (such as proliferating cells) within the duct of a breast belongs to either the epithelial or myoepithelial lineages. For example one can use ductal cannulation to expose luminal epithelial cells with a cell specific vector (such as replication-defective rAd) containing a reporter gene in order to evaluate the expansion of this cancer associated lineage. Alternatively, one can use ductal cannulation to expose myoepithelial cells with a cell specific vector (such as rAAV) containing a reporter gene in order to evaluate the expansion of this lineage. In this way a practitioner can determine whether a group of cells (such as a group of proliferating cells) is, like luminal epithelial cells, prone to cancer. In

addition, methods utilizing cell specific vectors containing reporter genes can be used to facilitate the assessment of the presence of occult cancer cells, thereby aiding in long term prognosis and treatment.

In a preferred embodiment of the invention, the method consists of determining the lineage of a cell in a ductal system in a mammary gland selected from the group consisting of a luminal epithelial cell and a myoepithelial cell, by using ductal cannulation to contact the cell with vector containing a reporter gene, wherein the vector selectively targets a molecule that is expressed on the luminal epithelial cell or the myoepithelial cell. As shown for example in Figures 3 and 4, the expression of the reporter gene can be used as an indication of transduction of the cell, with the specificity of the vector providing the information as to the cell lineage.

A wide variety of reporter genes and assays that are known in the art can be adapted to the diagnostic methods disclosed herein. For example, a reporter gene can encode an enzyme which produces colorimetric or fluorometric change in the host cell which is detectable by *in situ* analysis and which is a quantitative or semi-quantitative function of transcriptional activation. Exemplary enzymes include esterases, phosphatases, proteases (tissue plasminogen activator or urokinase) and other enzymes capable of being detected by activity which generates a chromophore or fluorophore as will be known to those skilled in the art. A preferred example is *E. coli* beta-galactosidase disclosed herein. This enzyme produces a color change upon cleavage of the indigogenic substrate indolyl-B-D-galactoside by cells bearing beta-galactosidase (see, e.g., Goring et al., Science, 235:456-458 (1987) and Price et al., Proc. Natl. Acad. Sci. U.S.A., 84:156-160 (1987)). This enzyme is preferred because the endogenous β -galactosidase activity in mammalian cells ordinarily is quite low, the analytic screening system using β -galactosidase is not hampered by host cell background.

Compositions of the Invention

In order to facilitate the utilization of vectors useful in gene therapy targeting cells of the breast duct, immortalized myoepithelial cell lines and transplantable xenografts were established from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-X; HMS-3, HMS-3X), breast (HMS-4, HMS-4X) and bronchus (HMS-6, HMS-6X) (M.D. Sternlicht et al. (1996) *In Vitro*, 32:550-563; M.D. Sternlicht et al. (1997) *Clin. Cancer Res.*, 3:1949-1958; Z. Shao et al. (1998) *Exper. Cell Res.*, 241:394-403). These cell lines and xenografts express identical myoepithelial markers as their *in situ* counterparts and display an essentially normal diploid karyotype. The myoepithelial cell lines and xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, I-1 antitrypsin, thrombospondin-1, soluble bFGF receptors,

and maspin. These suppressor molecules are well known in the art. (See generally, Sternlicht et al., Lab. Invest. 74(4):781-796 (1996) and Sternlicht et al., Med. Hypo. 48:37-46 (1997). In addition, for TIMP-1, see U.S. Pat. No. 5,595,885, for protease nexin-II, see U.S. Pat. No. 5,213,962, for α_1 -antitrypsin see U.S. Pat. No. 5,736,379, for thrombospondin-1 see U.S. Pat. No. 5,648,461, for bFGF receptors see 5,750,371, and for maspin see U.S. Pat. No. 5,470,970.

The human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion and endothelial migration and proliferation (angiogenesis) *in vitro*. The myoepithelial cell lines also inhibit breast carcinoma proliferation through an induction of breast carcinoma cell apoptosis, a phenomenon which occurs at high levels within DCIS (Z. Shao et al. (1998) Exper. Cell Res., 241:394-403). On the basis of immunoprecipitation studies, myoepithelial maspin seems to be the major effector molecule which inhibits invasion and thrombospondin-1 seems to be the major effector molecule which inhibits angiogenesis (S. Bodis et al. (1996) Cancer, 77:1831-1835). Myoepithelial nitric oxide seems to be the major effector molecule which inhibits breast carcinoma proliferation through its induction of apoptosis (Z. Shao et al. (1998) Multidiscip. Symposium on Breast Disease).

HMS-X, HMS-1, HMS-3, HMS-4 and HMS-6 have been deposited under the requirements of the Budapest Treaty on _____ with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 USA, and have been identified as ATCC Accession Nos. CRL 11899, CRL 11792 _____..

The present invention is further detailed in the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Example 1: Transfection of Cells with Adenovirus

This example relates to the finding is that primary breast ductal epithelial cells express CAR and are easily transduced with rAd whereas myoepithelial cells lack CAR and are completely resistant. CAR is thought to mediate, in part, the attachment of adenovirus to the cell membrane (J. Bergelson et al.

(1997) Science 275:1320-1323). In this context, one can use the rAd studies described below as an illustration of methods of the present invention.

rAd Studies: One can use a replication-defective rAd2 (see e.g. Hashimoto et al., Biochem. Biophys. Res. Comm., 240: 88-92 (1997)) with a β -galactosidase reporter and measure its ability to transduce epithelial cells compared to myoepithelial cells through *ex vivo* and *in vivo* approaches. In addition, one can use mastectomy specimens and deliver the rAd intraductally as was done with rAAV in Example 2 below. Because rAd does not integrate (unlike rAVV), the incubation period will probably be less (on the order of 72-96 hours). One can detect β -galactosidase by a substrate assay in frozen sections of the mastectomy specimens. One can then compare myoepithelial to epithelial colorimetric development. In parallel experiments one can deliver intraductally rAd to anesthetized rabbits and after a few days observe β -galactosidase activity in their breast sections. At this point, one can compare epithelial activity with myoepithelial activity. Even in situations using a replication-defective virus one can nevertheless measure adenovirus titers in the feces and urine of the rabbits by ELISA to evaluate how the myoepithelial layer offers a barrier to systemic infection.

Example 2: Transfection of Cells with Adeno-associated Virus

This example relates to the finding that unlike epithelial cells expression of CAR, myoepithelial cells, instead, express cell surface heparin sulfate proteoglycan (HS) and are easily transduced with rAAV. As discussed in Example 1 above, primary ductal epithelial cells have absence of this proteoglycan and are resistant to rAAV. Cell surface HS is thought to be a receptor for rAAV which determines transducibility (C. Summerford and R.J. Samulski, J. Virology 72:1438-1445). In this context, one can use the rAAV studies described below as an illustration of methods of the present invention. Up until we established that rAAV can be used to successfully transfect myoepithelial cells (HMS-1), our myoepithelial cell lines were completely resistant to transfection by all conventional means.

rAAV Studies: One can begin with rAAV (see e.g. Flannery et al., P.N.A.S., 94: 6916-6921, (1997)). This recombinant virus contains a CMV promoter and the human green fluorescent protein. One can then inject increasing titers of this virus together with a supravital dye, e.g. lymphazurin (the sentinel node dye) to help guide the injections into the tumor's center. One can then inject the myoepithelial xenografts disclosed herein (HMS-X, HMS-3X, HMS-4X, HMS-6X) of a size of 1 cm in

diameter and after 1-3 weeks extirpate these tumors, cut thin frozen sections and study the radial distribution of fluorescence in relation to the azure blue dye. As controls, one can use non-myoepithelial carcinoma tumors where one would expect transduction to be absent or minimal. One can then determine how rAAV transduction into myoepithelial cells followed by recombinant gene expression occurs *in vivo* by these direct tumoral injection studies. If positive results are obtained one can deliver rAAV intraductally via nipple cannulation into human mastectomy specimens *ex vivo* and into rabbit nipples *in vivo*. After 24 hours one can initiate organ culture explants of the mastectomies and then after several weeks observe fluorescence in myoepithelial cells compared to ductal epithelial cell. Since rabbits have eight nipples with 4 ductal systems each they provide a relatively simple animal model to test intraductal gene delivery. After three weeks one can sacrifice the rabbits, section their breasts and compare myoepithelial to epithelial fluorescence.

Example 3: Verifying observations concerning cell-specific targeting of recombinant adenovirus (rAd) and recombinant adeno-associated virus (rAAV) on epithelial and myoepithelial cells respectively.

Using *in vitro* experiments, we expand upon our earlier findings and outline the parameters that will facilitate *in vivo* practice of the invention. These parameters include determinations of dose (titer), frequency of administration, period of incubation, period of observation, etc. Controls for all of these correlative *in vitro* experiments include both non-reporter vector or no vector.

In addition, we rule out the possibility of a pseudotransduction phenomenon occurring in these examples (vector-mediated protein delivery rather than gene expression) especially in the rAAV experiments where this phenomenon has been observed by others. For example, in experiments where successful reporter gene product is observed, we verify that it is gene expression that we are delivering rather than just protein by carrying out a titer dilution experiment. Specifically, the effects of titer dilution should be to decrease protein staining in all cells if we are dealing with pseudotransfection; in true transfection there should be a decrease in the number of cells showing marker protein but the intensity of staining in the positive cells should not decrease. Our results establish that we are dealing with true transfection since we have made the latter observations. Furthermore in our *in vitro* rAAV experiments performed to date, the intensity of the reporter staining to human green fluorescent protein in the transfected HMS-1 cells increased at 3 weeks *versus* 1 week after transfection. This increase in reporter protein with increasing passage is indicative of true gene expression because rAAV delivered genes are always a bit slow to show full expression. If we were experiencing pseudotransfection (vector-mediated protein delivery), the reporter staining would *decrease* with passage.

In addition, we have backed up our proposed marker gene expression assays with a molecular genetic analysis showing the presence of virus by PCR studies.

Expanding upon our initial studies we conducted kinetic studies of transduction efficiency of reporter genes via their respective viral vectors into epithelial and myoepithelial cells *in vitro*.

In addition, we confirm the usefulness of E1A-deleted rAd containing two different promoters known in the art, i.e., CMV and RSV LTR.

Dose-response studies designed to determine whether there is a relationship between the vector multiplicity of infection (moi) and the efficiency of reporter gene (β -galactosidase) gene transfer into breast epithelial cells (HMEC) are included herein. Specifically, we observe that a moi of 10^2 will achieve 100% transduction efficiency. At the same time we monitored myoepithelial cells for resistance to rAd transduction and determined that their resistance is absolute. For example, they are completely resistant to transfection even with a moi of 10^4 .

We also conducted a time course of exposure to rAd over 1-10 hours to determine whether transfection efficiency is enhanced with a longer time of exposure. It is not.

In the case of rAd, we monitored reporter gene expression over time following infection to determine its rate of expression and its decay. We find a half life of expression of 48 hours. We repeated these *in vitro* studies with rAAV targeting myoepithelial cells. Since rAAV as opposed to rAd integrates into the genome but requires second strand synthesis during cell division before expression occurs (since rAAV is single stranded), we would expect a longer time course after transfection to observe expression but a shallower or no rate of decay. In this context, observe that maximal expression occurs at 3 weeks with negligible decay.

We also compare reporter expression in myoepithelial cells *versus* epithelial cells. We evaluate replication-competent and replication-enhanced viruses whose production of viral particles can be enhanced by promoter *cis/trans* interactions and monitor the lysis of epithelial cells and resistance to lysis of myoepithelial cells. Significant lysis is observed visually or by a dye exclusion method (trypan blue) or by a viability monitoring method like MTT. Similar kinetic experiments have been performed. In the case of the replication-enhanced rAd, exogenous agents such as dexamethasone and/or LPS designed to increase transcription from the enhancer/promoter being used (MUC1 and lactoalbumin) in our rAd-transfected breast epithelial cells (HMEC) have been monitored for effects on viral titer and a 10 fold increase has been observed with a 50% increase in cell lysis. Myoepithelial cells remain completely resistant to the effects of this replication-competent lytic virus.

Example 4: Verifying the Feasibility of Transfecting Suppressor Molecules Such as Maspin

We expand upon our *in vitro* experiments discussed above by using replication-defective rAd and rAAV that contain not reporter genes but native genes such as maspin and conduct similar kinetic studies. Specifically, we repeated all of the above studies that used reporter genes and instead used actual endogenous suppressor genes like maspin to establish that our approach is physiologically feasible.

Maspin transfection into myoepithelial cells via rAAV vectors The mechanisms of maspin's effects on invasion and motility inhibition are completely unknown. Maspin was initially identified by subtractive hybridization and the differential display method to identify candidate tumor suppressor genes that were defective in human breast carcinoma cells. It is interesting that even in those normal breast cell lines which were used to clone maspin and in those normal breast lines where it has been identified it is present only intracellularly and is not secreted. In contrast to epithelial cells, our myoepithelial cell lines do secrete maspin. The presence of secreted maspin in myoepithelial cells compared to normal epithelial cells and our results on maspin's invasion and motility inhibition of breast carcinoma cells support the hypothesis that maspin is acting as a paracrine and not an autocrine tumor suppressor.

Myoepithelial cells seem to have the unique ability to *secrete* this serpin. Therefore we exploited this property of myoepithelium by transfecting rmaspin into HMS-1 via rAAV *in vitro* to achieve maspin overexpression. rAAV containing the CMV promoter, for example, was used to modulate a full length maspin cDNA ligated into this vector. Levels of secreted rmaspin were determined by Western blot after several weeks. As shown in Figure 7, myoepithelial cells overexpressing maspin are more effective at blocking carcinoma cell invasion in Matrigel. Such Matrigel assays are well known in the art and provide a model for breast cancer systems (see e.g. Bae et al., Breast Cancer Res. Tret. 24(3): 241-55 (1993). Specifically, the effects of transfected myoepithelial clones expressing rmaspin in invasion inhibition assays show a 200% increase in inhibition of invasion. Consequently, these results demonstrate the feasibility of using the overexpression of maspin in myoepithelial as an *in vivo* gene therapy strategy.

Example 5: In Vivo Studies

The *ex vivo* findings discussed in Examples 1-4 above illustrate how one can, using an intraductal approach, selectively target and destroy breast epithelium *in vivo* yet spare the underlying

myoepithelium with rAd and/or alternatively selectively target the myoepithelium *in vivo* with rAAV and bolster its defense with genes such as maspin.

Specifically, using the preliminary *ex vivo* and *in vivo* studies disclosed herein, one can selectively target breast myoepithelium and breast epithelium with these vector-specific approaches. Therefore
5 one can proceed in the following manner: use a replication-competent lytic adenovirus or a replication-competent lytic adenovirus containing *cis* elements which stimulate replication when specific *trans* factors present in breast epithelium are encountered (a candidate *cis* element would be the lactoalbumin promoter or the MUC1 promoter). One can then evaluate the destruction of breast epithelium at risk for developing cancer and in effect perform a "prophylactic mastectomy" without having to remove the
10 breast. Whether the myoepithelial layer would serve as an effective barrier against systemic infection when a replication-competent lytic adenovirus virus is used can be determined by urine and feces ELISA. The resistance of myoepithelial cells to adenovirus infection makes it likely that this layer offers a defense against systemic infection. Alternatively one can use rAAV to selectively target myoepithelium and deliver to it a candidate gene such as maspin to bolster its defensive abilities.

What is claimed is:

1. A method of selectively transducing a cell within a mixed population of ductal epithelial and myoepithelial cells, comprising the step of contacting the cell with a cell specific vector that transduces the cell through a CAR molecule expressed by the ductal epithelial cell or a heparan sulfate proteoglycan molecule expressed by the myoepithelial cell.
2. The method according to claim 1 wherein the mixed population of ductal epithelial and myoepithelial cells is within a ductal system of a mammary gland.
3. The method according to claim 1, wherein the cell is contacted by ductal cannulation.
4. The method according to claim 1, wherein the cell is a ductal epithelial cell.
5. The method according to claim 4 wherein the vector induces cell death.
6. The method according to claim 5, wherein the vector is a replication competent adenovirus.
7. The method according to claim 6, wherein a control sequence in the replication-competent lytic adenovirus contains a *cis* element which stimulates adenovirus replication in the presence of a *trans* factor present in the ductal epithelial cell.
8. The method according to claim 7, wherein the *cis* element is selected from the group consisting of the lactoalbumin promoter and the MUC1 promoter.
9. The method according to claim 5, wherein the vector comprises a suicide gene selected from the group consisting of thymidine kinase and cytosine deaminase.
10. The method according to claim 1, wherein the cell is a myoepithelial cell.
11. The method according to claim 10 wherein the vector is a recombinant adeno-associated virus.
12. The method according to claim 10, wherein the vector comprises a gene encoding a polypeptide which inhibits the development of cancer.
13. The method according to claim 13, wherein a control sequence in the recombinant adeno-associated virus contains a *cis* element which modulates the expression of the gene in the presence of a *trans* factor present in the myoepithelial cell.

14. The method according to claim 12, wherein the polypeptide inhibits the proliferation of a ductal epithelial cell.
15. The method according to claim 12, wherein the polypeptide inhibits the invasion of a ductal epithelial cell.
- 5 16. The method according to claim 12, wherein the polypeptide inhibits endothelial migration.
17. The method according to claim 12, wherein the polypeptide inhibits angiogenesis.
18. The method according to claim 12, wherein the polypeptide increases the production of nitric oxide by the myoepithelial cell.
- 10 19. The method according to claim 12, wherein the polypeptide induces apoptosis in a ductal epithelial cell.
20. The method according to claim 12, wherein the polypeptide is selected from the group consisting of maspin, thrombospondin-1, TIMP-1, protease nexin-II, α -1 antitrypsin and soluble bFGF receptor.
- 15 21. A method of selectively transducing a cell in a ductal system in a mammary gland selected from the group consisting of a ductal epithelial cell and a myoepithelial cell, comprising the step of contacting, by ductal cannulation, the cell with a cell specific vector that selectively targets a molecule that is expressed on the ductal epithelial cell or the myoepithelial cell.
- 20 22. A method of selectively transducing a ductal epithelial cell in a ductal system in a mammary gland, comprising the step of contacting, by ductal cannulation, the cell with a vector that targets a CAR molecule expressed by the cell.
23. A method of selectively transducing a myoepithelial cell in a ductal system in a mammary gland, comprising the step of contacting, by ductal cannulation, the cell with a vector that targets a heparin sulfate proteoglycan molecule expressed by the cell.
- 25 24. A method of treating the ductal epithelium of a mammary gland for cancer of ductal epithelial cell origin, comprising the step of contacting, by ductal cannulation, a cell in a ductal system in a mammary gland selected from the group consisting of a ductal epithelial cell and a

myoepithelial cell, with a vector that enters the cell through a CAR molecule expressed by the ductal epithelial cell or a heparin sulfate proteoglycan molecule expressed by the myoepithelial cell, wherein the expression of the polynucleotides of the vector so transduced inhibits the formation of cancer of ductal epithelial cell origin.

5 25. The method according to claim 24, further comprising a treatment selected from the group consisting of surgical removal of the cancerous tissue, radiation therapy and chemotherapy.

26. A method of determining the identity of a cell in a ductal system in a mammary gland selected from the group consisting of a ductal epithelial cell and a myoepithelial cell, comprising:

10 (a) contacting the cell with vector comprising a reporter gene, wherein the vector selectively targets a molecule expressed:

(i) on the ductal epithelial cell; or

(ii) on the myoepithelial cell; and

(b) evaluating the expression of the reporter gene to determine the identity of the cell.

27. The myoepithelial cell line HMS-1 deposited as ATCC accession number CRL 11899.

15 28. The myoepithelial cell xenograft HMS-X deposited as ATCC accession number CRL 11792.

29. The myoepithelial cell line HMS-3 deposited as ATCC accession number ____.

30. The myoepithelial cell line HMS-4 deposited as ATCC accession number ____.

31. The myoepithelial cell line HMS-6 deposited as ATCC accession number ____.

GATES & COOPER
United States Patent Application
COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

COMPOSITIONS AND METHODS FOR INTRADUCTAL GENE THERAPY

The specification of which:

a. ☒ is attached hereto.

b. ☐ was filed on _____ as United States Application Number or PCT International Application Number _____ and was amended on _____ (if applicable), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT application having a filing date before that of the application on the basis of which priority is claimed:

a. ☒ no such applications have been filed.

b. ☐ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
OTHER FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or 365(c) of any PCT international application(s) designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose

material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. PARENT APPLICATION OR PCT PARENT NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)
60/116,470	20 JAN 99

I hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

George H. Gates	Registration No. 33,500
Victor G. Cooper	Registration No. 39,641
Anthony J. Orler	Registration No. 41,232
Karen S. Canady	Registration No. 39,927
William J. Wood	Registration No. 42,236
Jason S. Feldmar	Registration No. 39,187

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/ which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Gates & Cooper to the contrary.

Please direct all correspondence in this case to the firm of Gates & Cooper at the address indicated below:

GATES & COOPER
Howard Hughes Center
6701 Center Drive West, Suite 1050
Los Angeles, CA 90045

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(1)	Full Name Of Inventor	Family Name BARSKY	First Given Name SANFORD	Second Given Name H
	Residence & Citizenship	City Los Angeles	State or Foreign Country California	Country of Citizenship U.S.A.
	Post Office Address	Post Office Address 10422 Lindbrook Drive	City Los Angeles	State & Zip Code/Country California 90024/U.S.A.
Signature of Inventor(1):				Date:

(2)	Full Name Of Inventor	Family Name ALPAUGH	First Given Name MARY	Second Given Name L.
	Residence & Citizenship	City Los Angeles	State or Foreign Country California	Country of Citizenship U.S.A.
	Post Office Address	Post Office Address 10792 Wilkens Avenue	City Los Angeles	State & Zip Code/Country California 90024/U.S.A.
Signature of Inventor(2):				Date:

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) it establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) it refutes, or is inconsistent with, a position the applicant takes in:
 - (i) opposing an argument of unpatentability relied on by the Office, or
 - (ii) asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

- (1) each inventor named in the application;
- (2) each attorney or agent who prepares or prosecutes the application; and
- (3) every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

Abstract of the Disclosure

The present invention provides methods for the selective transduction of a cell in a ductal system in a mammary gland by contacting, via ductal cannulation, the cell with a vector that selectively targets the cell. In this context, the invention provides prophylactic and therapeutic methods of treating the ductal epithelium of the breast, for disease, in particular cancer. The present invention further provides diagnostic methods of determining the presence of disease in the ductal epithelium of the breast, in particular cancer.

"Express Mail" mailing label number EL30793940545

Date of Deposit JULY 20, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to:

Assistant Commissioner for Patents, Washington, D.C. 20231.

Adriane M. Baird
(printed name)

Adriane M. Baird
(Signature)

FIGURE 1

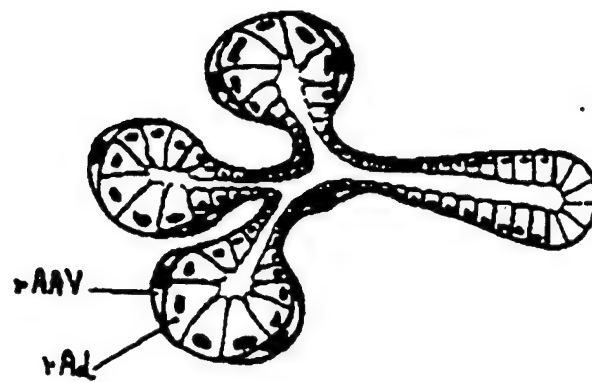


FIGURE 2



FIGURE 3

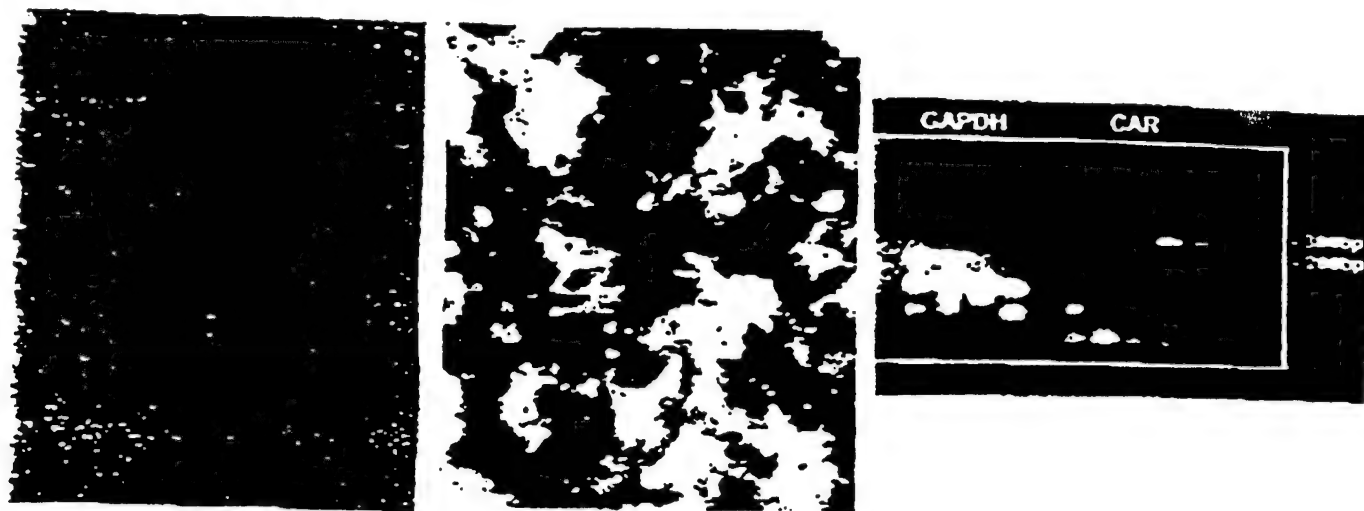


FIGURE 4

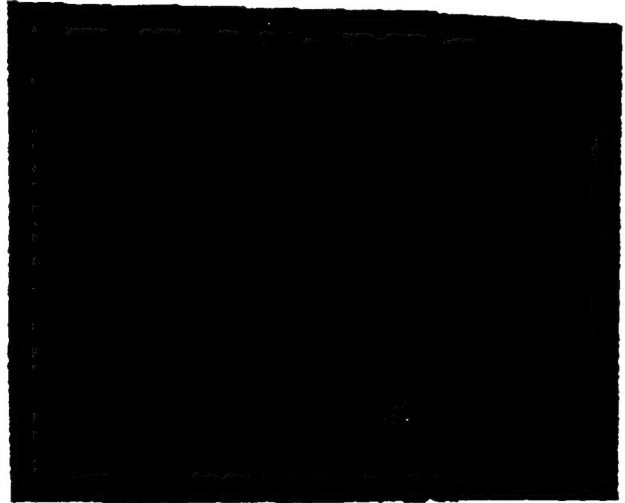
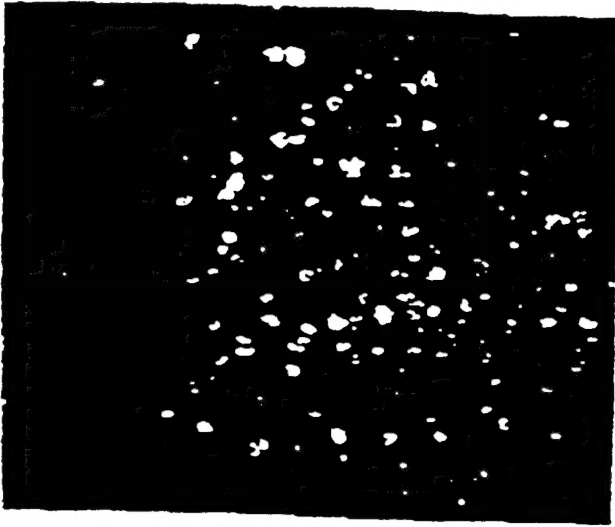
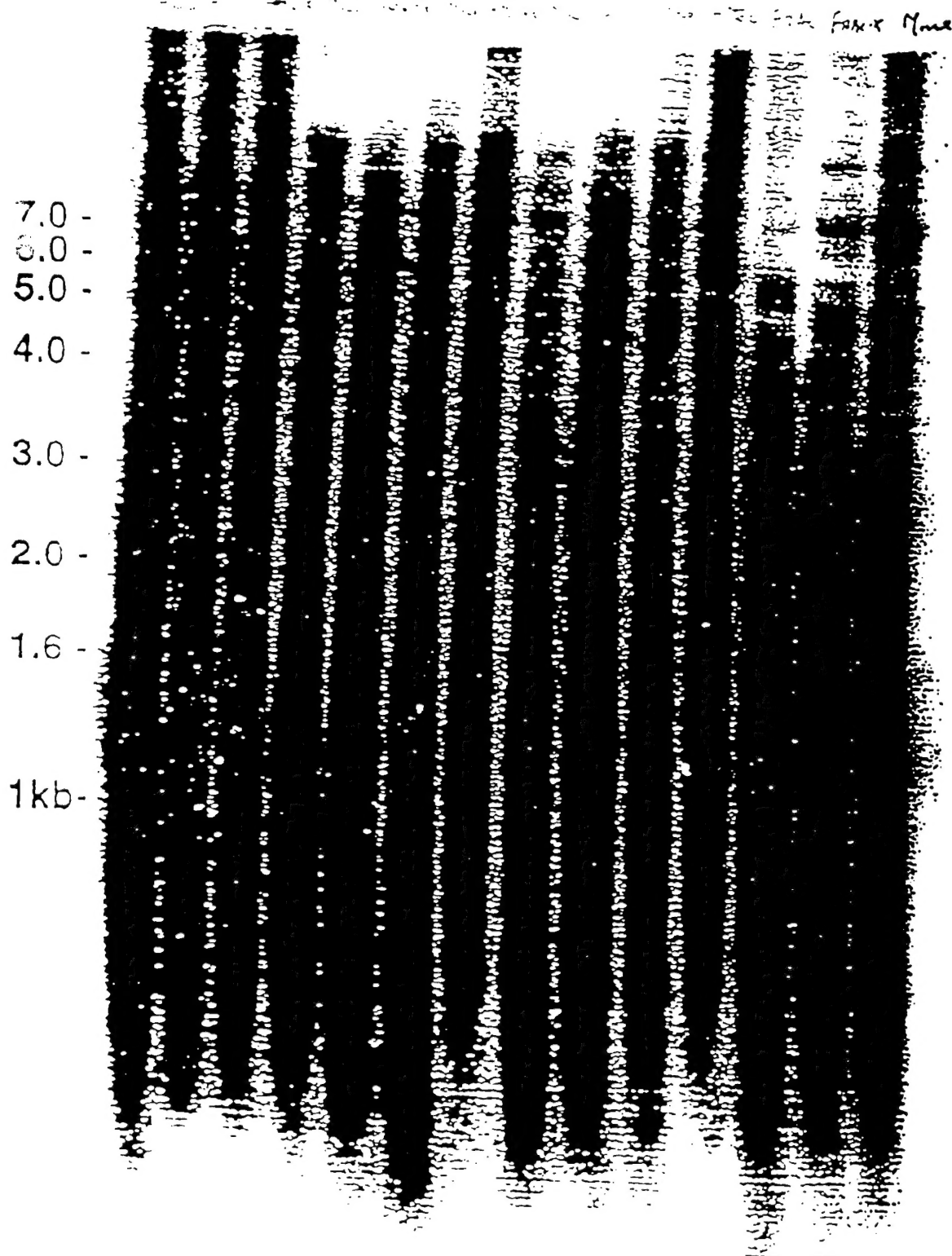
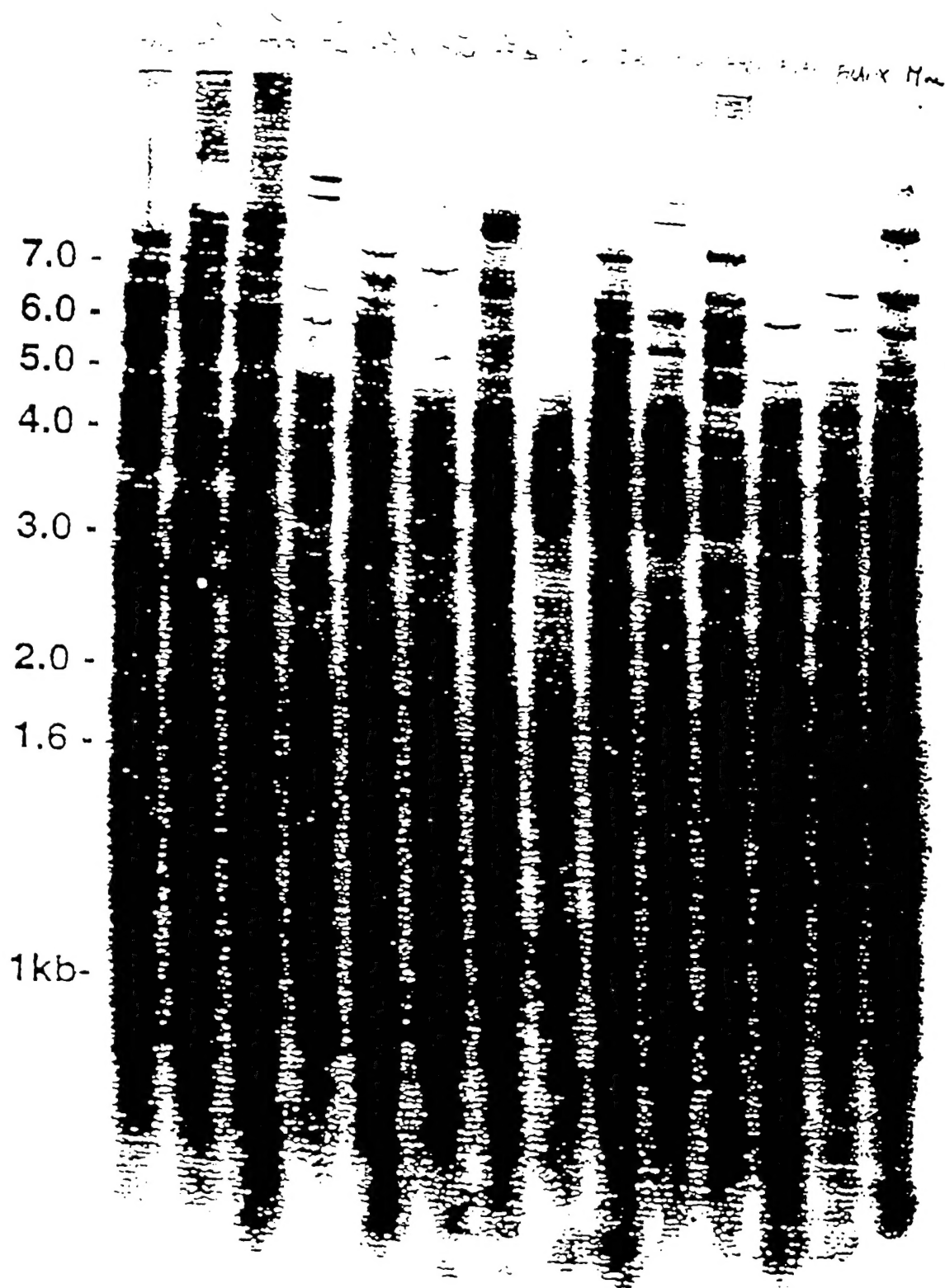


Figure 5 - Best Available Copy



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Figure 6 - Best Available Copy



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FIGURE 7

